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ORIGINAL ARTICLE

Carbon and nitrogen substrate utilization in the marine bacterium *Sphingopyxis alaskensis* strain RB2256

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Sphingopyxis alaskensis **is a marine member of the Alphaproteobacteria that is adapted to heterotrophic growth under nutrient-depleted (oligotrophic) conditions.** ***S. alaskensis*** **strain RB2256 is an ultramicrobacterium (cell volume** $<0.1\,\mu m^3$**), and has a genome size larger than that of the ultramicrobacterium '*Candidatus* Pelagibacter ubique' HTCC1062 (SAR11 clade of Alphaproteobacteria): 3.35 versus 1.31 Mbp. In this study, we investigate the carbon and nitrogen metabolism of strain RB2256 using an integrated approach that combines growth and enzyme assays, proteomics and genome analysis.** ***S. alaskensis*** **is able to use specific amino acids and putrescine as a sole carbon and nitrogen source, and higher energy-yielding substrates such as glucose and trehalose as carbon sources. Alanine, in particular, emerges as a very important substrate in** ***S. alaskensis*** **metabolism. In an oligotrophic environment where competition for nutrients is intense, our data support a simplified metabolism for** ***S. alaskensis*** **in which the fate of certain substrates is constrained, especially at the intersections of central carbon and nitrogen metabolism, in order to ensure optimal disposition of scarce resources. This is the first investigation of central metabolism for an oligotrophic ultramicrobacterium that possesses a relatively large genome size. In contrast to the behavior so far observed for SAR11 oligotrophic bacteria,** ***S. alaskensis*** **shows a physiological capacity to exploit increases in ambient nutrient availability and thereby achieve high-population densities.**

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Introduction

The Earth's oceans cover the majority of the surface of the planet, and have the highest cellular production rate of any ecosystem on the planet (Whitman *et al.*, 1998). In the open ocean, the vast majority of this production is carried out by bacteria, which, despite the bulk nutrient-depleted (oligotrophic) nature of the open ocean, can achieve densities in the order of $0.5–5 \times 10^5$ cells ml^{-1} (Schut *et al.*, 1997a; Whitman *et al.*, 1998). The majority of these bacteria are free-living (planktonic) forms that include the smallest of all living cells, with constant cell volumes of not more than $0.1\,\mu m^3$. These bacteria have been termed 'ultramicrobacteria', with cell volume ($<0.1\,\mu m^3$) as the defining criterion (Schut *et al.*, 1997a; Cavicchioli and Ostrowski, 2003). This is particularly useful for studies of natural communities as a variety of cell shapes is often encountered, and volume provides a measurement of cell size that is independent of morphology (Schut *et al.*, 1997a; Cavicchioli and Ostrowski, 2003). Ultramicrobacteria represent a major source of biomass and metabolic activity in oceanic ecosystems, and express higher metabolic activity per unit of volume of seawater than larger bacterial cells (Schut *et al.*, 1997a). As such, in marine oligotrophic environments, these small microbial cells play an essential role in regulating the accumulation, remineralization and transformation of the earth's largest pool of organic carbon. However, the physiology of ultramicrobacteria has remained largely uncharacterized, in a large part because of their resistance to cultivation.

The successful isolation and axenic cultivation of the ultramicrobacterium *Sphingopyxis alaskensis*

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strain RB2256 (formerly *Sphingomonas alaskensis*) has provided opportunities for studying oligotrophic growth and metabolism (Schut *et al.*, 1993, 1995, 1997a, b; Eguchi *et al.*, 1996; Fegatella *et al.*, 1998; Fegatella and Cavicchioli, 2000; Ostrowski *et al.*, 2001; Cavicchioli *et al.*, 2003). *S. alaskensis* cells are exceptionally small (cell volume $<0.1\,\mu m^3$), and remain essentially constant in size between starvation and growth conditions (Schut *et al.*, 1993, 1997a, b; Cavicchioli *et al.*, 2003). The reduced cell size of ultramicrobacteria provides high surface-to-volume ratios, which facilitates growth under oligotrophic conditions (Button, 1991), resistance to grazing by predatory zooplankton (González *et al.*, 1990), and the ability to partition biomass among a greater number of progeny from a given substrate pool (Fegatella *et al.*, 1998). *S. alaskensis* strain RB2256 was isolated from surface waters (10 m depth) of Resurrection Bay, Alaska, where it was isolated by an extinction dilution method as a numerically abundant bacterium ($>10^5$ cells ml^{-1}) (Schut *et al.*, 1993). *S. alaskensis* strain AF01 was also an abundant bacterial species sampled from another North Pacific site, in oligotrophic waters (350 m deep) off the coast of Japan (Eguchi *et al.*, 2001), and similar isolates have been obtained from the North Sea (Schut *et al.*, 1993). However, in contrast to '*Candidatus* Pelagibacter ubique' from the SAR11 clade (Giovannoni *et al.*, 2005), *S. alaskensis* does not seem to have been abundant in the samples taken for the Global Ocean Survey (which, however, does not include samples from the North Pacific) (Thomas *et al.*, 2007). Although it is presently unclear how widespread this species is in ocean waters, its isolation over a period spanning about a decade and abundance at the time of sampling in North Pacific locations indicates that *S. alaskensis* has the capacity to proliferate in oligotrophic marine waters.

The ability to grow slowly on low concentrations (nanomolar) of substrates and maintain a relatively constant cell size in the shift between starvation and growth conditions is an oligotrophic trait (Eguchi *et al.*, 1996; Schut *et al.*, 1997a, b; Fegatella and Cavicchioli, 2000; Cavicchioli *et al.*, 2003). On the basis of the Michaelis–Menten constants for substrate transport (K_t), and the available concentrations of dissolved free amino acids (DFAAs) in the ocean, *S. alaskensis* is predicted to be able to grow by using DFAAs at an *in situ* doubling time of 12 h to 3 days (Schut *et al.*, 1995), which compares favorably with measured doubling times for bacteria in oligotrophic waters of 5–15 days (Fuhrman *et al.*, 1989). These traits distinguish *S. alaskensis* from typical copiotrophic bacteria, which exhibit a 'feast-and-famine' response that entails rapid growth under nutrient-enriched conditions, but respond to nutrient depletion (including starvation) by undergoing reductive cell division to form resting-stage cells (Srinivasan and Kjelleberg, 1998; Cavicchioli *et al.*, 2003). Earlier studies on the physiology of *S. alaskensis* have shown a number of properties relevant to its oligotrophic ecology, including the ability to simultaneously take up mixed substrates, irrespective of concentration; a constitutive broad-specificity uptake system for amino acids; and inducible glucose uptake, with this substrate immediately converted to storage product, even during glucose-limiting growth (Schut *et al.*, 1995, 1997a). It has also been shown earlier that the slow growth rate of *S. alaskensis* ($<0.2\,h^{-1}$) (for example, compared with a copiotroph), even under favorable growth conditions (30 °C, millimolar nutrient levels), is not the result of insufficient ribosome synthesis, despite possessing only a single rRNA copy per genome (Fegatella *et al.*, 1998). Such experiments emphasize the physiological versatility of an ultramicrobacterium that is not confined to oligotrophic nutrient levels in order to grow, but also highlight gaps in our understanding of the critical aspects of *S. alaskensis* metabolism that may constrain growth, even under nutrient-sufficient conditions.

Earlier kinetic and metabolic data for *S. alaskensis* are consistent with a metabolism by which certain amino acids (such as alanine) represent important natural growth substrates, but glucose does not (Schut *et al.*, 1995, 1997a). Although they are relatively poor sources of carbon and energy, amino acids are ubiquitous in the open ocean, with alanine, glutamate, glycine and serine being major components of the DFAA reservoir (Lee and Bada, 1975, 1977; Andersson *et al.*, 1985; Ishida *et al.*, 1986; Eguchi and Ishida, 1990). Around 90% of the nitrogen requirements of bacteria in oligotrophic ocean waters are believed to be served by free ammonia and DFAAs (Keil and Kirchman, 1991). However, key aspects of the *S. alaskensis* metabolism remain unknown, including the processes by which ammonia and amino acids are metabolized by the cell. The aim of this study was to link the analysis of the genome sequence of *S. alaskensis* RB2256 to proteomic, growth and biochemical analyses in order to elucidate the carbon and nitrogen metabolism of this marine bacterium. One outcome of this approach was the identification of characteristics that helped to clarify how *S. alaskensis* could compete in oceanic environments, and how this bacterium compares with model oligotrophs such as '*Cand.* P. ubique' and specialist copiotrophs. This study is the first of this kind for a sphingomonad, a group known for its physiological and ecological versatility (White *et al.*, 1996; Cavicchioli *et al.*, 1999). Given the importance of ultramicrobacteria to carbon and nitrogen cycling in the open ocean, our study aims to provide important new insights into the evolution of metabolic strategies that have been selected in order for marine bacteria to proliferate in oceanic environments.

Materials and methods

Genome analysis

The complete and auto-annotated genome of *S. alaskensis* RB2256 was searched for genes of potential relevance to central carbon and nitrogen metabolism. Coding regions, automated annotation and manual curation of the *S. alaskensis* RB2256 genome were carried out as described for other JGI genomes (for example, Klotz *et al.*, 2006; Ivanova *et al.*, 2007). Functional assignments for genes were manually evaluated against experimental data from the literature and the confidence of each gene's predicted function was assigned an evidence rating (ER) value based on a system of manual annotation developed for *Methanococcoides burtonii* (Allen *et al.*, 2009): ER1, *S. alaskensis* protein had been experimentally characterized; ER2, the most closely related functionally characterized ortholog share $\geqslant 35\%$ sequence identity along the entire length of the protein; ER3, the most closely related functionally characterized homolog shares $<35\%$ sequence identity along the length of the protein, but all required motifs/domains for function are present; ER4, an experimentally characterized full-length homolog is not available but conserved protein motifs or domains can be identified; ER5 (hypothetical protein), no functionally characterized homolog can be found, and no characterized protein domains above the Pfam and InterProScan cut-off thresholds can be identified. The genome sequences of '*Cand*. P. ubique' HTCC1062 (Rappé *et al.*, 2002; Giovannoni *et al.*, 2005), *Silicibacter pomeroyi* DSS-3 (González *et al.*, 1999; Moran *et al.*, 2004), *Photobacterium angustum* S14 (Humphrey *et al.*, 1983; Kjelleberg *et al.*, 1993) and *Pseudoalteromonas haloplanktis* TAC125 (Médigue *et al.*, 2005; Stocker *et al.*, 2008) were used for comparative analysis as representatives of marine bacteria with diverse strategies for nutrient acquisition. '*Cand.* P. ubique' HTCC1062 is a non-motile oligotroph isolated from the coast of Oregon (Rappé *et al.*, 2002). It is the first cultured member of the ubiquitous SAR11 clade and has an exceptionally small genome (1.31 Mb) for a free-living bacterium, and represents an example of extensive genomic streamlining in a marine bacterium (Giovannoni *et al.*, 2005). *S. pomeroyi* DSS-3 (*Roseobacter* group, Alphaproteobacteria) was isolated off the coast of Georgia (González *et al.*, 1999) and is thought to have a trophic strategy involving association (including direct attachment) with algal blooms (Moran *et al.*, 2004). Its genome has an exceptionally high number of ABC transporter genes, but no identifiable genes associated with chemotaxis (Moran *et al.*, 2004). *P. haloplanktis* TAC125 (Gammaproteobacteria) was isolated in Antarctic coastal waters (Médigue *et al.*, 2005), and is a marine bacterium that specializes in exploiting ephemeral nutrient pulses and plumes through chemotaxis (Jackson, 1989; Stocker *et al.*, 2008). *Photobacterium* (formerly *Vibrio*) *angustum* S14 (Gammaproteobacteria) was isolated from coastal waters in southeastern Australia, and is a model copiotrophic bacterium that undergoes reductive cell division and mounts a strong starvation-induced stress protection response (Humphrey *et al.*, 1983; Kjelleberg *et al.*, 1993).

Growth conditions and preparation of cell extract

S. alaskensis RB2256 was grown in artificial seawater (ASW) medium (Eguchi *et al.*, 1996) at 30 °C in 250 ml side-armed conical flasks on a rotary shaker at 150 r.p.m. Growth was monitored at 433 nm on various carbon and nitrogen compounds (stipulated in Results). The concentrations of substrates used in the study were at levels higher than typically found in seawater; this was for two reasons. First, conditions that promote strong *S. alaskensis* growth were used to gauge the activities of enzymes that were potentially important to carbon and nitrogen metabolism. This was important in order to ascertain which metabolic pathways associated with ammonia assimilation and amino acid catabolism were represented in *S. alaskensis*, especially considering that genes for certain metabolic enzymes seem to be absent from the *S. alaskensis* genome. Second, the ability of *S. alaskensis* to use substrates for growth is dependent upon appropriate transport mechanisms; using millimolar levels ensured that intracellular fluxes of these substrates were sufficient, even if transport mechanisms were inefficient in certain cases.

To prepare crude cell extract for enzyme assays, *S. alaskensis* was grown in ASW medium with the addition of various carbon and nitrogen sources (stipulated in Results). Cultures were grown at 30 °C and harvested at mid-logarithmic phase and centrifuged at 10 000 *g* for 20 min at 4 °C. The cell pellet was washed once in 50 mM Tris-HCl buffer (pH 7.6) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and stored at −20 °C until required. The cell pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.6) containing 1 mM EDTA and 1 mM phenylmethanesulfonylfluoride (PMSF). The cells were disrupted on ice by sonication for 3 min or by a passage through a French pressure cell (prechilled, at 18 000 lb in^{-2}) and centrifuged at 21 000 × *g* for 30 min at 4 °C. The resulting supernatant solution was used to assay enzyme activity.

Enzyme assays

Activities of glutamate dehydrogenase (GDH; EC 1.4.1.2 (NAD)/EC 1.4.1.4 (NADP)), alanine dehydrogenase (AlaDH; EC 1.4.1.1), and glutamate synthase (GOGAT) (EC 1.4.1.13) were measured spectrophotometrically from the rate of NAD(P)H oxidation or NAD(P)$^+$ reduction at 340 nm at room temperature ($\varepsilon_{340} = 6.22$ mM^{-1} cm^{-1}). Values were corrected for endogenous rates measured in the absence of

substrates. The low NADH oxidase activity present in the crude extract was determined with appropriate reagent blanks. One unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADPH or NADH or reduction of 1 μmol of $NAD(P)^+$ per min. The amount of protein in the crude extract was estimated at 595 nm using the method of Bradford (1976) with bovine serum albumin as the standard. All results are averages of values derived from at least two independent experiments.

NAD(P)H-GDH (aminating) reaction mixture contained 100 mM Tris-HCl buffer, pH 8.0 (pH 7.5 for NADH-GDH assay), 50 mM NH_4Cl, 15 mM 2-oxoglutarate, 0.2 mM NADH or NADPH, and crude extract. The $NAD(P)^+$-GDH (deaminating) activities were measured in the reaction mixture containing 100 mM Tris-HCl buffer, pH 9.0, 100 mM glutamate, 1 mM $NAD(P)^+$ and crude extract. NADH-AlaDH (aminating) assay mixture contained 100 mM Tris-HCl buffer, pH 8.8, 50 mM NH_4Cl, 20 mM pyruvate, 0.2 mM NADH and crude extract. The NAD^+-AlaDH (deaminating) assay mixture contained 100 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) pH 10.0, 40 mM alanine, 1 mM NADH and crude extract. Binding order of substrates in AlaDH activity was determined by adding reagents sequentially to the reaction mixture containing reaction buffer and crude extract. NADPH-GOGAT reaction mixture contained 100 mM Tris-HCl buffer, pH 7.6, or 100 mM K-buffer, pH 7.6, and 10 mM glutamine, 2 mM 2-oxoglutarate (neutralized with KOH), 0.2 mM NADPH and crude extract.

Glutamine synthetase (GS; EC 6.3.1.2) activity in *S. alaskensis* crude extracts was measured by its transferase and biosynthetic hydroxamate activities, based on Ertan (1992a). One unit of GS activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol γ-glutamyl hydroxamate per min. In this study, GS levels were measured by transferase assay because the state of adenylation has little effect on the γ-glutamyl transferase activity (Shapiro and Stadtman, 1970). The effect of the glutamate analog L-methionine-S-sulfoximine (MSO) (2 mM), an irreversible inhibitor of GS (Ronzio *et al.*, 1969), was also tested for its effect on *S. alaskensis* growth (Ertan, 1992b).

Glutaminase (EC 3.5.1.2) catalyzes the reversible hydrolysis of glutamine to glutamate, yielding ammonium. Glutaminase activity was measured in both forward and reverse directions, by coupled reaction of bovine liver GDH (Sigma, St Louis, MO, USA, G-2501) and γ-glutamyl hydroxamate assays, respectively (Prusiner *et al.*, 1972, 1976).

Alanine aminotransferase (AlaAT; EC 2.6.1.2) activity was assayed spectrophotometrically by monitoring NADH oxidation at 340 nm for 3 min. Activity in the pyruvate-to-alanine direction was determined by coupling the reaction to NADH oxidation by GDH (Bergmeyer, 1983). The reaction was assayed in a final 2.4-ml mixture containing 5 U of GDH (Sigma G2501), 100 mM Tris-HCl pH 8.0, 50 mM NH_4Cl, 0.18 mm NADH, 10 mM pyruvate, 0.025 mM pyridoxal phosphate, 15 mM glutamate and crude extract. One unit of AlaAT was defined as the amount catalyzing the formation of 1.0 μmol of product per min at 30 °C.

α,α-Trehalose phosphorylase (EC 2.4.1.64) activity was measured in the direction of trehalose phosphorolysis as described earlier (Aisaka and Masuda, 1995), with the exception that the trehalose concentration was 200 mM. One unit of enzyme activity was defined as the amount of the enzyme that liberates 1 μmol of glucose per min.

Proteomics

In separate proteomic studies focusing on the response of *S. alaskensis* to growth at different temperatures (L Ting *et al.*, not shown), 2135 proteins (66% proteome coverage) were identified from cells harvested at late-logarithmic phase (OD_{433} of 0.3) grown in ASW medium containing 3 mM glucose and 9.4 mM ammonia. Briefly, cell lysates were separated using gel-based fractionation, followed by in-gel tryptic digestion, and the resulting peptides were analyzed using nanoLC and data-dependent tandem mass spectrometry (MS/MS) against a *S. alaskensis* genome database. In this study, the proteomics analysis was limited to proteins that were potentially relevant to carbon and nitrogen metabolism in *S. alaskensis*.

Results

Nutrient uptake

The *S. alaskensis* genome contains seven putative ATP-binding cassette (ABC) transport systems for nutrient uptake: two for sugar transport, and one each for general amino acids (Aap-type), polyamines (putrescine/spermidine), phosphate, Fe^{3+}-siderophore and molybdate. Despite high proteomics coverage, only a single complete ABC transporter system (phosphate uptake) was detected during growth of *S. alaskensis* on glucose and ammonia (Table 1), and no uptake system responsible for glucose uptake could be identified. The high concentrations (millimolar) of glucose and ammonia used in the growth media may have suppressed expression of energetically expensive ABC transport systems responsible for importing other substrates (such as amino acids). In addition to primary (ABC-mediated) uptake, glucose has been shown to be imported in *S. alaskensis* by a second mechanism that is independent of group translocation (Schut *et al.*, 1995). Both TRAP (tripartite ATP-independent periplasmic) transporter genes and those for PTS (phosphotransferase system) transport are absent from the *S. alaskensis* genome. Symporter and permease genes for the uptake of sugars, carboxylic acids, amino acids and oligopeptides are present in

Table 1 Sphingopyxis alaskensis genes and putative function for proteins involved in carbon and nitrogen pathways, showing proteins detected by proteomics

Transport (uptake)	
Sala_0822	Phosphate uptake regulator protein (phoU) (ER2)
Sala_0823	ABC phosphate transport system, ATP-binding component (pstB) (ER2)
Sala_0824	ABC phosphate transport system, permease component (pstA) (ER3)
Sala_0825	ABC phosphate transport system, permease component (pstC) (ER3)
Sala_0826	ABC phosphate transport system, phosphate-binding component (pstS) (ER3)
Sala_0748	ABC sugar transport system, solute-binding component (malE) (ER3)
Sala_0749	ABC sugar transport system, permease component (malD/malG) (ER3)
Sala_0750	ABC sugar transport system, permease component (malG) (ER3)
Sala_0751	ABC sugar transport system, ATP-binding component (malK) (ER2)
Sala_1021	ABC sugar transport system, permease component (araQ/malD/malG/lacG) (ER3)
Sala_1022	ABC sugar transport system, permease component (araP/malD/malG/lacF) (ER3)
Sala_1023	ABC sugar transport system, solute-binding component (malE) (ER2)
Sala_1025	ABC sugar transport system, ATP-binding component (malK/lacK) (ER2)
Sala_1114	ABC spermidine/putrescine transport system, ATP-binding component (potA) (ER2)
Sala_1115	ABC spermidine/putrescine transport system, solute-binding component (potC/potE) (ER2)
Sala_1116	ABC spermidine/putrescine transport system, permease component (potB) (ER2)
Sala_1117	ABC spermidine/putrescine transport system, solute-binding component (potF) (ER3)
Sala_2412	ABC general L-amino acid transport system, ATP-binding component (aapP) (ER2)
Sala_2414	ABC general L-amino acid transport system, permease component (aapQ) (ER2)
Sala_2415	ABC general L-amino acid transport system, solute-binding component (aapJ) (ER2)
Sala_0550	ABC molybdate transport system, solute-binding component (modA) (ER3)
Sala_0551	ABC molybdate transport system, permease component (modB) (ER2)
Sala_0552	ABC molybdate transport system, ATP-binding component (modC) (ER2)
Sala_1001	ABC Fe^{3+}-siderophore transport system, ATP-binding component (fecE/hmuV) (ER3)
Sala_1002	ABC Fe^{3+}-siderophore transport system, permease component (fecD/hmuU) (ER3)
Sala_1003	ABC Fe^{3+}-siderophore transport system, solute-binding component (hmuT/btuF) (ER3)
Sala_0661	Glutamate/aspartate or carboxylic acid symporters (gltP/gltT or dctA) (ER3)
Sala_0959	Glutamate/aspartate or carboxylic acid symporters (gltP/gltT or dctA) (ER3)
Sala_1456	Carboxylic acid symporter (dctA) (ER2)
Sala_1446	Sugar symporter (melB) (ER3)
Sala_3075	Sugar symporter (melB) (ER3)
Sala_1704	Amino acid or polyamine permease (ER4)
Sala_2384	Amino acid or polyamine permease (ER4)
Sala_3167	Amino acid or polyamine permease (ER4)
Sala_0954	Peptide permease (ER3)
TCA cycle (including glyoxylate bypass and PEP-pyruvate-oxaloacetate anaplerotic node)	
Sala_0525	Pyruvate dehydrogenase E1 component, α subunit (pdhA) (EC 1.2.4.1) (ER2)
Sala_0526	Pyruvate dehydrogenase E1 component, β subunit (pdhB) (EC 1.2.4.1) (ER2)
Sala_1235	Dihydrolipoamide acetyltransferase component, pyruvate dehydrogenase complex (E2) (pdhC) (EC 2.3.1.12) (ER2)
Sala_1237	Dihydrolipoyl dehydrogenase component, pyruvate dehydrogenase complex (pdhD) (E3) (EC 1.8.1.4) (ER2)
Sala_0829	Citrate synthase 1 (gltA) (EC 2.3.3.1) (ER2)
Sala_2096	Aconitrate hydratase 1 (acnA) (EC 4.2.1.3) (ER2)
Sala_1964	Isocitrate dehydrogenase (NADP) (icd) (EC 1.1.1.42) (ER2)
Sala_2228	2-Oxoglutarate dehydrogenase E1 component (sucA) (EC 1.2.4.2) (ER2)
Sala_2227	Dihydrolipoyllysine-residue succinyltransferase component, 2-oxoglutarate dehydrogenase complex (E2) (sucB) (EC 2.3.1.61) (ER2)
Sala_2225	Dihydrolipoyldehydrogenase component, 2-oxoglutarate dehydrogenase complex (E3) (odhL) (EC 1.8.1.4) (ER2)
Sala_2229	Succinyl-CoA synthetase, α subunit (sucD) (EC 6.2.1.5) (ER2)
Sala_0176	Succinyl-CoA synthetase, β subunit (sucC) (EC 6.2.1.5) (ER2)
Sala_3032	Succinate dehydrogenase flavoprotein subunit (sdhA) (EC 1.3.5.1) (ER2)
Sala_2234	Succinate dehydrogenase iron-sulfur subunit (sdhB) (EC 1.3.5.1) (ER2)
Sala_3030	Succinate dehydrogenase cytochrome *b* subunit (sdhC) (EC 1.3.5.1) (ER2)
Sala_3031	Succinate dehydrogenase membrane anchor subunit (sdhD) (EC 1.3.5.1) (ER3)
Sala_3152	Fumarate hydratase class II (fumC) (EC 4.2.1.2) (ER2)
Sala_2230	Malate dehydrogenase (NAD) (mdh) (EC 1.1.1.37) (ER2)
Sala_1811	Acetyl-coenzyme A synthetase (acsA) (EC 6.2.1.1) (ER2)
Sala_0943	Isocitrate lyase (aceA) (EC 4.1.3.1) (ER3)
Sala_1935	Malate synthase G (glcB) (EC 2.3.3.9) (ER2)
Sala_3081	Phosphoenolpyruvate carboxylase (ppc) (EC 4.1.1.31) (ER2)
Sala_2046	Phosphoenolpyruvate carboxykinase (ATP) (pckA) (EC 4.1.1.49) (ER2)
Sala_2792	Malate dehydrogenase (oxaloacetate-decarboxylating) (NADP) ('malic enzyme') (maeB) (EC 1.1.1.40) (ER2)
Sala_1610	Pyruvate phosphate dikinase (ppdK) (EC 2.7.9.1) (ER2)

Table 1 Continued

Entner–Doudoroff pathway, gluconeogenesis and pentose phosphate pathway	
Sala_1110	Glucokinase (ATP-D-glucose-6-phosphotransferase) (glk) (EC 2.7.1.2) (ER2)
Sala_0304	Putative bifunctional enzyme containing *N*-terminal trehalose phosphorylase (EC 2.4.1.64) and *C*-terminal β-phosphoglucomutase (EC 5.4.2.6) (ER3)
Sala_0190	Glucose-6-phosphate 1-dehydrogenase (zwf) (EC 1.1.1.49) (ER2)
-	6-Phosphogluconolactonase (EC 3.1.1.31) – not present (spontaneous hydrolysis?)
Sala_0191	6-Phosphogluconate dehydratase (edd) (EC 4.2.1.12) (ER2)
Sala_0192	2-Dehydro-3-deoxy-phosphogluconate aldolase (eda) (EC 4.1.2.14) (ER2)
Sala_1319	Glyceraldehyde-3-phosphate dehydrogenase (gap) (EC 1.2.1.12)) (ER2)
Sala_1318	Phosphoglycerate kinase (pgk) (EC 2.7.2.3) (ER2)
Sala_2829	Phosphoglycerate mutase (gpmA) (EC 5.4.2.1) (ER2)
Sala_0523	Enolase (eno) (2-phosphoglycerate dehydratase) (EC 4.2.1.11) (ER2)
Sala_0341	Pyruvate kinase (pyk) (EC 2.7.1.40) (ER2)
Sala_1172	Triosephosphate isomerase (tpiA) (EC 5.3.1.1) (ER2)
Sala_1317	Fructose-bisphosphate aldolase, class 1 (edA) (EC 4.1.2.13) (ER2)
Sala_1215	Glucose-6-phosphate isomerase (pgi) (EC 5.3.1.9) (ER2)
Sala_2107	Fructose-1,6-bisphosphatase, class II (glpX) (EC 3.1.3.11) (ER2)
Sala_0114	6-Phosphogluconate dehydrogenase (decarboxylating) (gnt2) (EC 1.1.1.44) (ER3)
Sala_1843	6-Phosphogluconate dehydrogenase, NAD-binding (ER3)
Sala_0089	Ribose-5-phosphate isomerase A (rpiA) (EC 5.3.1.6) (ER2)
Sala_0792	Ribose-5-phosphate isomerase B (rpiB) (EC 5.3.1.6) (ER2)
Sala_3128	Ribulose-phosphate 3-epimerase (rpe)(EC 5.1.3.1) (ER2)
Sala_1320	Transketolases (tklB) (EC 2.2.1.1) (ER2)
Sala_1807	Transaldolase (tal) (EC 2.2.1.2) (ER2)
PHA synthesis and degradation	
Sala_3158	Acetyl-CoA acetyltransferase (phaA) (EC 2.3.1.9) (ER2)
Sala_2255	Acetoacetyl-CoA reductase (phaB) (EC 1.1.1.36) (ER2)
Sala_0505	PHA polymerase (phaC) (EC 2.3.1.-) (ER2)
Sala_1984	PHA depolymerase (phaZ) (EC 3.1.1.75) (ER4)
Sala_0504	Phasin (phaP) (ER4)
Nitrogen metabolism/utilization	
Sala_2327	Ammonium permease (amtB) (ER2)
Sala_2149	Nitrogen regulatory protein EI-Ntr (ptsP) (ER2)
Sala_2836	Nitrogen regulatory protein EIIA-Ntr (ptsN) (ER2)
Sala_2051	Nitrogen regulatory protein EIIA-Ntr (ptsN) (ER3)
Sala_2052	Nitrogen regulatory protein NPr (ptsO) (ER2)
Sala_2837	RNA polymerase, σ54 subunit (rpoN) (ER3)
Sala_2999	RNA polymerase, σ54 subunit (rpoN) (ER2)
Sala_2326	Nitrogen regulatory protein PII (glnK) (ER2)
Sala_0148	Nitrogen regulatory protein PII (glnB) (ER2)
Sala_2787	PII uridylyl-transferase/uridylyl-removing enzyme (UT-UR) (glnD) (ER2)
Sala_1277	tRNA-dihydrouridine synthase (dusB) (ER2) (ER2)
Sala_1276	Nitrogen-specific signal transduction histidine kinase (ntrB) (ER2)
Sala_1275	Nitrogen assimilation regulatory protein, transcriptional regulator (ntrC) (ER2)
Sala_1274	Nitrogen-specific signal transduction histidine kinase (ntrY) (ER2)
Sala_1273	Nitrogen assimilation regulatory protein, transcriptional regulator (ntrX) (ER2)
Sala_2185	Glutamine-synthetase adenylyltransferase (glnE) (EC 2.7.7.49) (ER2)
Sala_1262	Nitrate regulatory protein (nasR) (ER4)
Sala_1263	Nitrate transporter protein (nrtA) (ER3)
Sala_1264	Nitrate transporter protein (nasA) (ER2)
Sala_1268	Assimilatory nitrate reductase, α-subunit (apoprotein) (nasC) (EC 1.7.1.1) (ER2)
Sala_1267	Assimilatory nitrite reductase (NAD(P)H), small subunits (nasE) (EC 1.7.1.4) (ER2)
Sala_1266	Assimilatory nitrite reductase (NAD(P)H), large subunit (nasB/nasD) (EC 1.7.1.4) (ER2)
Sala_1269	Uroporphyrinogen-III C-methyltransferase (nasF) (EC 2.1.1.107) (ER2)
Sala_0149	Glutamine synthetase 1 (glnA) (EC 6.3.1.2) (ER2)
Sala_2140	Glutamate synthase (GOGAT), alpha subunit (gltB) (EC 1.4.1.13) (ER2)
Sala_2138	Glutamate synthase (GOGAT), beta subunit (gltD) (EC 1.4.1.13) (ER2)
Sala_2771	Glutamate dehydrogenase (NADP) (gdhA) (EC 1.4.1.4) (ER2)
Sala_2258	Glutamate dehydrogenase (NAD) (gdh-1) (EC 1.4.1.2) (ER3)
Sala_0558	Alanine dehydrogenase (ald) (EC 1.4.1.1) (ER2)
Sala_1227	Alanine racemase (alr) (EC 5.1.1.1) (ER2)
Sala_1867	Glycine cleavage system, P protein (=glycine dehydrogenase (decarboxylating)), α subunit (gcvP) (EC 1.4.4.2) (ER2)
Sala_1868	Glycine cleavage system, P protein (=glycine dehydrogenase (decarboxylating)), β subunit (gcvPA) (EC 1.4.4.2) (ER2)
Sala_1870	Glycine cleavage system, H protein (gcvH) (ER2)
Sala_1871	Glycine cleavage system, T protein (gcvT) (EC 2.1.2.10) (ER2)
Sala_2225	Glycine cleavage system, L protein (lpd) (EC 1.8.1.4) (ER2)
Sala_0616	3-Phosphoglycerate dehydrogenase (serA) (EC 1.1.1.95) (ER2)
Sala_0617	Phosphoserine aminotransferase (serC) (EC 2.6.1.52) (ER2)

Table 1 Continued

Nitrogen metabolism/utilization	
Sala_1468	Phosphoserine phosphatase (serB) (EC 3.1.3.3) (ER2)
Sala_0496	Threonine aldolase (ltaE) (EC 4.1.2.5) (ER2)
Sala_0791	Serine hydroxymethyltransferase (glyA) (EC 2.1.2.1) (ER2)
Sala_0292	L-Serine deaminase (sdaA) (EC 4.3.1.17) (ER2)
Putrescine catabolism	
Sala_1118	γ-Glutamylputrescine synthetase (EC 6.3.1.11) (puuA) (ER2)
Sala_1121	γ-Glutamylputrescine synthetase (EC 6.3.1.11) (puuA) (ER2)
Sala_1120	γ-Glutamylputrescine oxidase (EC 1.4.3.-) (puuB) (ER2)
Sala_2454	γ-Glutamyl-γ-amino-butyraldehyde dehydrogenase (puuC) (EC 1.2.1.-) (ER2)
Sala_1111	γ-Glutamyl-γ-amino-butyraldehyde hydrolase (puuD) (EC 3.5.1.94) (ER2)
Sala_1112	γ-Glutamyl-γ-amino-butyraldehyde aminotransferase (puuE/gabT) (EC 2.6.1.19) (ER3)
Sala_2116	Succinate-semialdehyde dehydrogenase (gabD) (EC 1.2.1.16) (ER2)
Motility and chemotaxis	
Sala_2903	Flagellar biosynthesis protein (flhA) (ER2)
Sala_2907	Flagellar distal basal body ring component protein (flaD) (ER4)
Sala_2908	Flagellar basal-body rod protein (flgC) (ER2)
Sala_2909	Flagellar hook capping protein/basal-body rod modification protein (flgD) (ER2)
Sala_2911	Flagellar basal-body rod protein (flgF) (ER2)
Sala_2912	Flagellar basal-body rod protein (flgG) (ER2)
Sala_2913	Flagellar L-ring protein (flgH) (ER2)
Sala_2914	Flagellar P-ring protein (flgI) (ER2)
Sala_2916	Flagellar hook-associated protein 1 (flgK) (ER3)
Sala_2917	Flagellar hook-associated protein 3 (flgL) (ER3)
Sala_2918	Flagellar motility protein (motA) (ER3)
Sala_2921	Flagellar protein (fliS) (ER3)
Sala_2922	B-type flagellar hook-associated protein 2 (fliD) (ER3)
Sala_2924	Flagellar biosynthetic protein (fliR) (ER3)
Sala_2925	Flagellar biosynthetic protein (fliQ) (ER3)
Sala_2926	Flagellar biosynthetic protein (fliP) (ER2)
Sala_2928	Flagellar motor switch protein (fliN) (ER2)
Sala_2930	Flagellar basal body-associated protein (fliL) (ER3)
Sala_2931	Flagellar hook-length control protein (ER4)
Sala_2934	Flagellar biosynthetic protein (fliH) (ER4)
Sala_2935	Flagellar motor switch protein (fliG) (ER3)
Sala_2936	Flagellar M-ring protein (fliF) (ER3)
Sala_2937	Flagellar hook-basal body complex protein (fliE) (ER2)
Sala_2938	Flagellin (fliC) (ER2)
Sala_0234	Methyl-accepting chemotaxis protein (ER3)
Sala_0556	Methyl-accepting chemotaxis protein (ER3)
Sala_1641	Methyl-accepting chemotaxis protein (ER3)
Sala_1736	Chemotaxis protein methyltransferase, CheR-type (ER3)
Sala_1737	Chemotaxis response regulator protein, CheB (ER3)
Sala_1738	Chemotaxis response regulator protein, CheY (ER2)
Sala_1739	Chemotaxis protein, CheW (ER3)
Sala_1740	Chemotaxis protein, CheA (ER2)

The function of each protein was inferred through manual annotation, and each annotation received an error rating (ER) according to the confidence of the identification (ER2-ER4 in decreasing confidence of predicted function). Cultures were grown on an artificial seawater-based medium that also contained glucose (3 mM) and ammonia (9.4 mM). Proteins (identified by locus tag) that were detected using proteomic analysis (pooled from 20 runs) are shaded according to the number of mass spectrometry experiments in which they were detected: 16–20 (dark gray); 1–15 (light gray); not detected in any experiment (white).

the genome, although substrate specificity for these systems was often difficult to infer (Table 1). Such transporters are presumed to include the mechanisms for the uptake of acetate, glutamate and other growth substrates not imported by primary transport.

S. alaskensis grows poorly or does not grow at all with particular carbon or nitrogen compounds (Table 2, and described below). This may be because of inefficient or unavailable transport mechanisms, such as for fructose (consistent with lack of PTS transport), pyruvate, tricarboxylic acid (TCA) intermediates and glutamate. However, in other cases, this could also reflect an absence of appropriate enzymes (for example, gluconokinase/gluconate).

Central carbon metabolism

S. alaskensis is an obligate heterotroph, which is consistent with the absence of any genes associated with known autotrophic pathways (for example,

Table 2 Growth response of *Sphingopyxis alaskensis* RB2256 using various carbon- and/or nitrogen-containing compounds as growth substrates

Carbon source	*Nitrogen source*	*Growth*
Glucose (3 mM)	NH_4Cl (0.9 mM)	++
Glucose (3 mM)	NH_4Cl (9 mM)	+++
Glucose (3 mM)	$(NH_4)_2SO_4$ (9 mM)	+++
Glucose (3 mM)	KNO_3 (5 mM)	++
Glucose (3 mM)	Glutamate (5 mM)	++
Glucose (3 mM)	Glutamate (10 mM)	++
Glucose (3 mM)	Glutamine (5 mM)	+++
Glucose (3 mM)	Alanine (5 mM)	++
Glucose (3 mM)	Glycine (10 mM)	+++
Glucose (3 mM)	Serine (10 mM)	+++
Glucose (3 mM)	Putrescine (5 mM)	+++
Trehalose (1 mM)	NH_4Cl (0.9 mM)	+++
Trehalose (1 mM)	NH_4Cl (9 mM)	+++
Trehalose (1 mM)	Glutamate (5 mM)	++
Glycine (10 mM)	NH_4Cl (9 mM)	NG
Pyruvate (5 mM)	NH_4Cl (6 mM)	+
Succinate (5 mM)	NH_4Cl (9 mM)	+
Acetate (6 mM)	NH_4Cl (9 mM)	+
Maltose (5 mM)	NH_4Cl (9 mM)	++
Sucrose (5 mM)	NH_4Cl (9 mM)	++
Putrescine (10 mM)	NH_4Cl (9 mM)	+++
Alanine (5 mM)		++
Alanine (10 mM)		++
Glutamate (5 mM)		NG
Glutamate (10 mM)		++
Glutamine (10 mM)		++
Serine (10 mM)		NG
Putrescine (10 mM)		+++
Luria-Bertani (LB) medium		+++

Growth was monitored for 10 days by measuring optical densities at 433 nm, and ranked as weak (<0.3; +), moderate (between 0.3 and 0.6; ++), good (<0.6; +++), or none at all (NG). In addition to the tabulated data, there was no growth on the following carbon sources (5 mm), with NH_4Cl (9 mm) as the nitrogen source: fructose, D-mannitol, D-sorbitol, D-xylose, citrate, gluconate and glucuronate.

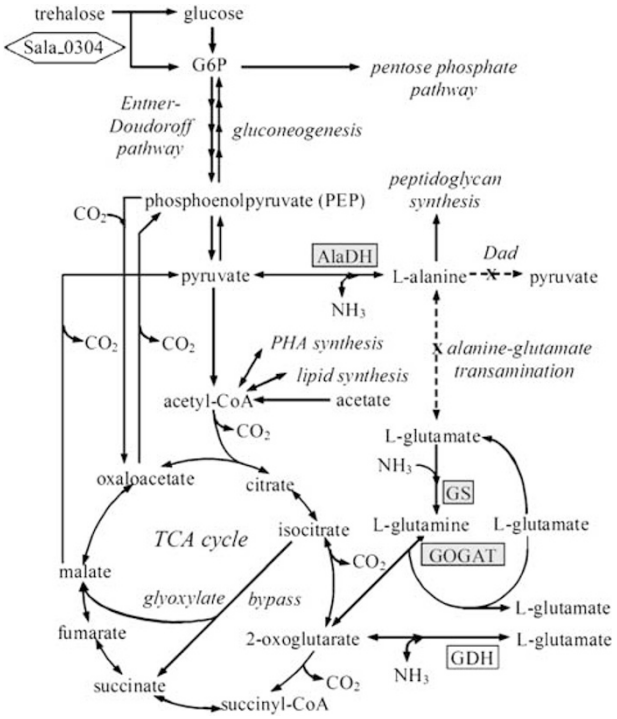

Figure 1 Simplified pathway of central carbon and nitrogen metabolism in *Sphingopyxis alaskensis* RB2256, based on genomic, proteomic and biochemical data. Pathways that exist in *S. alaskensis* are shown as solid arrows; pathways that are absent are shown with stippled arrows. Enzymes involved directly in ammonia assimilation are boxed: GS (glutamine synthetase), GOGAT (glutamate synthase), AlaDH (alanine dehydrogenase) and GDH (glutamate dehydrogenase). The major ammonia assimilatory enzymes in *S. alaskensis* are shaded in gray. Also shown is a unique putative bifunctional enzyme involved in trehalose cleavage, which is inferred to have both trehalose phosphorylase and β-phosphoglucomutase activity.

Calvin–Benson–Bassham cycle; reductive TCA cycle). *S. alaskensis* has a complete set of genes for a functional TCA cycle (Figure 1). The ability to grow on acetate as the sole carbon source, combined with predicted genes for isocitrate lyase and malate synthase, points to the operation of the glyoxylate bypass in *S. alaskensis* (Tables 1 and 2). *S. alaskensis* has a pathway for polyhydroxyalkanoic acid (PHA) synthesis and degradation, and several PHA pathway enzymes were identified in the proteomic data (Table 1). PHA synthesis in this species (26% cellular dry weight; Godoy *et al.*, 2003) is indicative of *S. alaskensis* diverting surplus carbon and energy to storage material. Further, operation of the glyoxylate bypass in *S. alaskensis* would allow acetyl-coenzyme A (acetyl-CoA) derived from PHA degradation to be fed into the TCA cycle without subsequent loss of carbon.

Genome analysis indicates that, as in other sphingomonads, *S. alaskensis* uses the Entner–Doudoroff pathway for glucose catabolism (Vartak *et al.*, 1995; Seo *et al.*, 2004; Table 1). The only missing gene is 6-phosphogluconolactonase; neither the *pgl* or *ybhE* forms (Zimenkov *et al.*, 2005) are present, indicating that hydrolysis of the ester linkage may occur spontaneously (non-enzymatically) (Kupor and Fraenkel, 1972). For the gluconeogenic direction, it is to be noted that the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate seems to be catalyzed by a GlpX-type (type II) fructose-1,6-bisphosphatase (FBPase II) (Donahue *et al.*, 2000). The auto-annotated genome sequence has two candidates for 6-phosphogluconate dehydrogenase. The protein Sala_1843, but not Sala_0114, was identified by proteomics to be expressed in *S. alaskensis* during growth on glucose (Table 1). This suggests that Sala_1843 is a 6-phosphogluconate dehydrogenase involved in the pentose phosphate cycle. Proteomic data also suggest that operation of the Entner–Doudoroff pathway is accompanied by expression of 'gluconeogenic' enzymes (Table 1). Such enzymes may be involved in the generation of fructose-6-phosphate through isomerization of glucose-6-phosphate, or through the two-step conversion from glyceraldehyde-3-phosphate. The latter could also be converted to dehydroxyacetone phosphate for synthesis of glycerol for lipid backbones.

Genes for pyruvate kinase and pyruvate dehydrogenase complex are present to facilitate the entry of acetyl-CoA into the TCA cycle. Anaplerotic replenishment of oxaloacetate from phosphoenolpyruvate (PEP) is presumably carried out by PEP carboxylase. PEP can be supplied from the TCA cycle intermediates malate and oxaloacetate using either PEP carboxykinase, or a combination of malic enzyme and pyruvate phosphate dikinase.

Trehalose utilization

Trehalose can be used as a carbon source as readily as glucose (Tables 2 and 3), but the only identifiable gene in the *S. alaskensis* genome for the cleavage of trehalose encodes a fusion protein (Sala_0304) with a trehalose phosphorylase or trehalase amino domain and a β-phosphoglucomutase carboxy domain. Trehalose phosphorylase activity was 125 mU mg^{-1} for *S. alaskensis* cells grown in 2 mM trehalose + 9 mM NH_4Cl, compared with 11 and 8 mU mg^{-1} for cells grown in 3 mM glucose + 5 mM glutamate and 3 mM glucose + 10 mM glutamine, respectively. The enzyme assays show that minimal glucose was liberated in the absence of phosphate, indicating that *S. alaskensis* does not possess trehalase activity. This putative bifunctional enzyme suggests that two steps are catalyzed by a single protein: trehalose cleavage into glucose and glucose 1-phosphate, and subsequent glucose 1-phosphate conversion into glucose 6-phosphate. Higher trehalose phosphorylase activity occured in trehalose-grown cells, with low but reproducibly detectable levels present in the absence of trehalose indicating that this enzyme is constitutively expressed; the latter is supported by identification of trehalose phosphorylase from proteomics data of glucose-grown cells (Table 1).

Ammonia and nitrate assimilation

S. alaskensis can use both NH_4Cl and KNO_3 as the sole source of nitrogen in defined medium in combination with different carbon sources (Table 2). The ability to assimilate nitrate is concordant with the presence of a gene cluster that encodes proteins responsible for nitrate transport and nitrate reduction to ammonia. These proteins were not detected during growth on media containing ammonia (9.4 mM), suggesting that nitrate utilization machinery is not expressed when ammonia (rather than nitrate) is the nitrogen source (Table 1). A predicted ammonia transporter gene (*amtB*) is present in the genome, and the protein was detected during growth on ammonia (Table 1).

The *S. alaskensis* genome sequence has a complete set of Ntr regulatory genes, most of which were detected by proteomics analysis (Table 1). Enzyme activity data indicate that the GS in *S. alaskensis* is not repressed by high concentrations of exogenous ammonia (Table 3). Nitrogen metabolism is governed by a phosphorylation cascade involving PtsP that phosphorylates PtsO and the regulator PtsN, and controls expression of all σ^{54}-dependent genes (Reizer *et al.*, 1992; Commichau *et al.*, 2006). The detection of PtsNOP and Ntr proteins indicates that nitrogen metabolism is tightly regulated in *S. alaskensis*.

S. alaskensis has a number of genes for enzymes implicated in ammonia assimilation, including GS (type 1), GOGAT (α and β subunits), GDH (NADP-dependent) and AlaDH. The auto-annotated *S. alaskensis* genome originally included three genes annotated as GS, but we re-annotated two (Sala_1118, Sala_1121) as putative γ-glutamylputrescine synthetase genes, involved in putrescine degradation (Table 1). There are two predicted GDH genes in *S. alaskensis*: NADP-dependent GDH

Table 3 Specific activities for enzyme assays carried out for *Sphingopyxis alaskensis* RB2256, for glutamine synthetase (GS), alanine dehydrogenase (AlaDH) and glutamate dehydrogenase (GDH)

Carbon and nitrogen sources in artificial sea water medium	*Doubling time (h)*	*Specific activity (mU mg^{-1})*						
		GS[a]	*AlaDH NADH*	*AlaDH NAD^+*	*GDH NADPH*	*GDH $NADP^+$*	*GDH NADH*	*GDH NAD^+*
3 mM Glucose + 0.5 mM NH_4Cl	7–8	400	150	13	5	5	0	0
3 mM Glucose + 9 mMNH_4Cl	6–7	540	160	22	64	60	ND	ND
6 mM Glucose + 9 mM NH_4Cl	ND	720	330	35	24	15	ND	ND
6 mM Glucose + 18 mM NH_4Cl	ND	1390	540	49	25	41	0	0
12 mM Glucose + 9 mM NH_4Cl	ND	960	110	19	2	17	16	18
2 mM Trehalose + 9 mM NH_4Cl	6–7	1350	980	71	10	25	ND	ND
3 mM Glucose + 5 mM KNO_3	7–8	1640	25	8	91	35	11	0
3 mM Glucose + 5 mM Glutamate	6–7	1320	24	5	30	55	9	12
3 mM Glucose + 5 mM Glutamine	5	420	120	6	60	36	5	2
3 mM Glucose + 5 mM L-alanine	7–8	2580	850	86	21	70	ND	ND
10 mM Alanine	9–10	640	3650	350	130	90	ND	ND
Luria–Bertani (LB) medium	5–6	33	1190	1190	200	20	440	73

Abbreviation: ND, not determined.
[a]Transferase activity.

(Sala_2771) and NAD-dependent GDH (Sala_2258). No glutaminase activity was detected in *S. alaskensis*, which is consistent with the absence of a glutaminase gene.

Attempts to measure GOGAT in *S. alaskensis* crude extract were unsuccessful; activities were below detectable levels, despite testing different disruption techniques and buffers. We attribute the lack of activity to the instability of the enzyme under experimental conditions, as encountered by other studies (for example, Brown and Dilworth, 1975; Boland and Benny, 1977; Chen *et al*., 1987). The absence of glutaminase activity rules out the possibility of a competing two-step reaction occurring in place of GOGAT reaction that would involve the liberation of ammonia from glutamine through glutaminase and subsequent assimilation of ammonia through GDH to produce glutamate.

GS activity was detected across all growth conditions, and showed a trend of increasing activity with increasing exogenous ammonia concentration (Table 3). The transferase assay for GS biosynthetic activity was successful, but the hydroxamate assay did not yield activity. The transferase assay facilitates an examination of the effect of different carbon and nitrogen sources on repression/derepression of GS levels, and provides a good assessment of GS levels under all growth conditions (the biosynthetic hydroxamate assay only measures unadenylated GS). pH optimum of GS transferase activity was 6.8 in imidazole-HCl buffer. GS activity was low when alanine was used as the sole carbon and nitrogen source, but high when alanine was combined with glucose in the medium. Thus, the presence of alanine in the growth medium elicited a strong response from both GS (when alanine was combined with glucose) and AlaDH (when used as the sole source of carbon and nitrogen). The glutamate analog MSO inhibited *S. alaskensis* growth (determined by maximum OD_{433}) by approximately 50% compared with untreated cultures. However, adding glutamine to the MSO-containing medium restored growth. The data indicate that, although GS-GOGAT is the major pathway for ammonia assimilation in *S. alaskensis*, ammonia assimilation can proceed when GS is inhibited.

Levels of AlaDH activity generally rose with increasing ammonia concentration (Table 3), and the data strongly support a predominantly assimilatory role for AlaDH in *S. alaskensis*. Proteomics data show that AlaDH is one of the major proteins in *S. alaskensis* grown on alanine-free media containing glucose and ammonia (Table 1). The enzyme activity data show that *S. alaskensis* AlaDH is NAD-dependent in both oxidative and reductive directions (there is no NADP-dependent activity). The K_m values of *S. alaskensis* AlaDH for L-alanine and ammonia were 5.5 and 4.9 mM, respectively. The K_m value for ammonia is among the lowest recorded for bacterial AlaDH for which a role in ammonia assimilation has been shown (cf. rhizobial bacteroids: 5–9 mM (Smith and Emerich, 1993; Allaway *et al*., 2000); *Anabaena cylindrica*: <8 mM (Rowell and Stewart, 1975); *Rhodobacter capsulatus*: 16 mM (Caballero *et al*., 1989a); *Streptomyces clavuligerus*: 20 mM (Aharonowitz and Friedrich, 1980)). In the reductive amination direction, substrates were bound to *S. alaskensis* AlaDH in the order NH_4^+, NADH, pyruvate. In the reverse direction, there was no order of substrate binding. Thus, ammonia can bind the active site without the requirement for binding of other substrates, in contrast to other AlaDH enzymes, in which NH_4^+ binding is dependent upon the binding of NADH or NADH/pyruvate complex (for example, Grimshaw and Cleland, 1981; Smith and Emerich, 1993). For *S. alaskensis*, reductive aminating activity was significantly higher than oxidative deamination under most growth conditions (Table 3), and the optimum pH was closer to physiological pH: 8.8–9.0 for amination, compared with 10.0 for deamination. In all, 20 mM alanine inhibited NADH-AlaDH activity by around 50%, and 50 mM NH_4Cl inhibited NAD^+-AlaDH activity by around 90%. These data indicate that product inhibition by reaction products is one of the regulatory mechanisms for *S. alaskensis* AlaDH activity, in both directions.

AlaDH activities were influenced by the nature and concentration of the carbon and nitrogen sources. Activities rose with increasing ammonia concentration (consistent with an assimilatory role), and increasing glucose concentrations (likely because of increased availability of pyruvate) up to 12 mM, when AlaDH activity was low. The low activity at high concentrations is consistent with earlier findings that showed that high glucose represses alanine oxidation (Schut *et al*., 1995). The low AlaDH activities in response to nitrate and glutamate indicate that, in contrast to GS and NADP-GDH, AlaDH seems to have a limited role in assimilating endogenously generated ammonia, such as resulting from nitrate reduction or glutamate catabolism. Higher AlaDH activity in response to glutamine is not unexpected, given that glutamine typically represses GS activity.

Both NADP- and NAD-dependent GDH activities were detected, although those for NAD-GDH were low (Table 3). The optimum pH values for reductive and oxidative activities of NADP-GDH were 8.0 and 8.5, respectively. For NAD-GDH, the reductive amination of 2-oxoglutarate had a pH optimum of 9, and the oxidative deamination of glutamate had a broad optimum spanning pH 8.5–10.0. The K_m value of NADP-GDH for glutamate was 7.7 mM. GDH clearly has a limited role in exogenous ammonia assimilation in *S. alaskensis*. NADP-GDH was not responsive to increasing ammonia concentration in growth medium, which would be otherwise expected for an ammonia assimilatory enzyme. The better growth of *S. alaskensis* on glutamine compared with ammonia as the sole nitrogen source is also consistent with low assimilatory GDH activity

(Rossi *et al*., 1989; Patriarca *et al*., 2002). However, GDH seems to play a role in the incorporation of endogenously generated ammonia. The highest NADP-GDH values were found with alanine as the sole carbon and nitrogen source, or when nitrate was the nitrogen source (Table 3). This is interpreted as a response to the high levels of intracellular ammonia liberated from alanine by AlaDH, or by the reduction of nitrate. Nevertheless, GDH catabolic function is presumably sufficient for *S. alaskensis* to grow on glutamate and glutamine, with the latter catabolized by the combined action of GOGAT and GDH.

In summary, GS and AlaDH were found to be the principal assimilatory enzymes under all conditions tested in our enzyme assays. Proteomic data showed GS, GOGAT and AlaDH to be among the most frequently detected proteins during growth on high-ammonia media. These data underscore the important role for AlaDH in ammonia assimilation in *S. alaskensis* and, conversely, the relatively minor role of GDH. GS is a major ammonia assimilatory enzyme, but there is no evidence that GS activity is repressed by high ammonia levels in the medium, even up to 18 mM. Nevertheless, some regulation of nitrogen metabolism is apparent, given that GS and AlaDH show contrasting responses to individual carbon and nitrogen sources. The combination of a rich carbon source (glucose) and a poor nitrogen source (nitrate, glutamate, alanine) resulted in high GS activity, presumably a result of derepression of GS. This response contrasts with AlaDH activity, which showed an opposite response to high glucose and/or poor nitrogen sources (nitrate, glutamate and glutamine, but not alanine). The high GS activities in response to nitrate and glutamate presumably result from low intracellular ammonia concentrations, which limits glutamine production, and thereby derepresses GS.

Amino acid metabolism

Growth data show that *S. alaskensis* is evidently capable of utilizing alanine, glutamate and glutamine as the sole source of carbon and nitrogen (Table 2). In addition, alanine, glutamate, glutamine, glycine and serine can each be used as a nitrogen source when glucose is present in the medium. A gene for D-amino acid dehydrogenase (DadA) could not be identified in the genome, indicating that AlaDH is responsible for alanine catabolism, as well as being an assimilatory enzyme (see the section 'Ammonia and nitrate assimilation'). AlaDH has been observed to substitute for the Dad system when overexpressed in a rhizobial strain that lacks the Dad system, which is normally the principal mechanism for alanine catabolism (Allaway *et al*., 2000; Lodwig *et al*., 2004). A catabolic role for AlaDH in *S. alaskensis* is consistent with the observation that elevated AlaDH values were attained for cells grown on alanine. The presence of nitrate, glutamate and glutamine in the media resulted in low AlaDH activities, as did high glucose (12 mM).

The NAD-GDH belongs to the 'large GDH' subfamily, which has been ascribed a role in glutamate catabolism (Miñambres *et al*., 2000). Owing to the apparent absence of a glutamate decarboxylase gene in the *S. alaskensis* genome glutamate cannot be catabolized by the γ-aminobutyrate (GABA) shunt. The putative NAD-GDH was among the most frequently detected proteins during growth on high-ammonia media (Table 1), with endogenous glutamate generated from GS-GOGAT able to serve as a substrate for catabolic GDH. Glutamate supplied in the media could be used as a sole carbon and nitrogen source for *S. alaskensis* growth when present at 10 mM, but not at 5 mM (Table 2). Glutamate could also serve as a nitrogen source at 5 mM when accompanied by glucose or trehalose as a carbon source (Table 2). Earlier, it was found from a mixture of 10 amino acids (1 mM each; including alanine, glutamate, glutamine and serine) that glutamate was the only amino acid not to be utilized by *S. alaskensis* (Schut *et al*., 1995). These data indicate that the utilization of glutamate as a growth substrate is dependent upon glutamate concentration and/or the identity of the (other) carbon source. Given that the single amino acid ABC transport system in *S. alaskensis* is not receptive to glutamate, this amino acid must be imported by other mechanisms that require high solute concentrations, or an energy-rich carbon source.

GDH activity in *S. alaskensis* was not induced by the presence of glutamate in the media (even in the presence of NAD^+ or $NADP^+$). For *S. alaskensis*, weak catabolic GDH that is not induced by glutamate may correlate with the absence of an ABC transport system capable of importing glutamate (see the section 'Nutrient uptake'). Overall, this response to glutamate is in marked contrast to the response of *S. alakensis* to alanine, which is imported by active ABC transport, and results in elevated AlaDH levels. Nevertheless, GDH catabolic function is presumably sufficient for *S. alaskensis* to grow on glutamate.

No AlaAT activity was observed for *S. alaskensis*, which is consistent with the absence of a gene responsible for this transamination enzyme. Several aminotransferase genes are present, but only two (apartate aminotransferase, branched-chain-amino acid aminotransferase) seem to encode enzymes capable of mediating transaminations among the 20 common amino acids. The inability of *S. alaskensis* to grow on glycine as a carbon source, despite growth on alanine, is consistent with the absence of an alanine-glyoxylate aminotransferase gene. There is no aspartate decarboxylase gene for the synthesis of aspartate through the carboxylation of alanine. Thus, there is no available mechanism for the direct interconversion of alanine and glutamate, which can therefore only occur indirectly though central carbon metabolism in order for either

to serve as both a carbon and nitrogen source for growth.

Glycine and serine can each serve as a nitrogen source for the growth of *S. alaskensis*, but not as a carbon source. Genes for the components of the glycine cleavage enzyme complex are present in the *S. alaskensis* genome. This complex liberates ammonia as a nitrogen source, but the carbon skeleton is first decarboxylated, then directed to C1 metabolism, precluding its use as a carbon source for growth. No deaminating glycine dehydrogenase gene is present. Genes are present for the interconversion of glycine and serine or threonine, the synthesis of serine from 3-phosphoglycerate and the deamination of serine; all the corresponding proteins were identified by proteomics analysis (Table 1). Thus, the inability to use glycine or serine as a carbon source is interesting in light of the genomic and proteomic data, which indicate that *S. alaskensis* is metabolically equipped to degrade these amino acids to pyruvate and ammonia. L-serine deaminase, which was expressed in *S. alaskensis* under conditions (high glucose) for which serine deamination should not be necessary, may therefore have a metabolic role that is not directly related to growth on amino acids (Su *et al.*, 1989).

Putrescine catabolism

Strong growth was observed on putrescine when it was supplied either as the sole carbon and nitrogen source, or as a nitrogen source in combination with glucose (Table 2). The genome has a gene cluster for the uptake (ABC transport) and utilization of putrescine. This pathway catabolizes putrescine by way of glutamylated intermediates, and generates ammonia and succinate, which enters the TCA cycle (Kurihara *et al.*, 2005). Many of the enzymes of this pathway were detected by proteomics from cells growing on glucose (Table 1), suggesting that this pathway may be constitutively expressed in *S. alaskensis*.

Discussion

Substrate preference and utilization

S. alaskensis is versatile in its ability to utilize carbon and nitrogen compounds as substrates for growth. Ammonia and nitrate can be used as nitrogen sources, despite the high energetic cost associated with nitrate utilization. This is in contrast to a number of other marine bacteria that lack the ability to assimilate nitrate, including '*Cand.* P. ubique' (for example, Dufresne *et al.*, 2003; García-Fernández *et al.*, 2004; Giovannoni *et al.*, 2005). *S. alaskensis* was isolated as an abundant bacterium from the oceanic waters of the North Pacific (Button *et al.*, 1993; Schut *et al.*, 1993; Eguchi *et al.*, 2001), which indicates that it contributed to the assimilation of inorganic nitrogen in these waters at the time of sampling. Our data particularly highlight the important role of alanine in this process, as it is a major product of ammonia and nitrate assimilation by *S. alaskensis*.

Certain amino acids (alanine, glutamate, glutamine) and putrescine can each be used by *S. alaskensis* as sole carbon and nitrogen sources, and these substrates are ubiquitous at nanomolar concentrations in seawater (Lee and Bada, 1975, 1977; Eguchi and Ishida, 1990; Nishibori *et al.*, 2001, 2003). Extracytoplasmic solute capture is extremely important under oligotrophic conditions (Schut *et al.*, 1995, 1997a; Giovannoni *et al.*, 2005; Sowell *et al.*, 2009). *S. alaskensis* has only a single amino acid ABC transport system (Table 4) that has earlier been found to be constitutive for alanine, in contrast to the inducible uptake of glucose (Schut *et al.*, 1995). The uptake of alanine was also found to be competitively inhibited by several amino acids (including glycine, although weakly), but not glutamate, glutamine or serine (Schut *et al.*, 1995). Our data indicate that the earlier observation that 1 mM glutamate is not used by *S. alaskensis* (Schut *et al.*, 1995) is likely a consequence of insufficient transport into the cytoplasm, rather than an absence of glutamate catabolic activity. The available data therefore indicate that alanine is a very important natural growth substrate for *S. alaskensis*, but that glutamate and serine are not. Furthermore, AlaDH activities were elevated in response to alanine, in contrast to low and non-inducible GDH activities in response to glutamate (Table 3). Thus, alanine is singled out by *S. alaskensis* as a preferred growth substrate, which is a further refinement of the preference for amino acids earlier documented for alphaproteobacteria (Cottrell and Kirchman, 2003; Malmstrom *et al.*, 2004). This may represent a key aspect of the oligotrophic ecology of *S. alaskensis*, as an example of simplification at the level of metabolic processing. Pyruvate is an important intermediate positioned at the nexus of several metabolic pathways (TCA cycle, gluconeogenesis, fatty acid synthesis, PHA synthesis), and therefore potentially a more versatile intermediate than 2-oxoglutarate, the deamination product of glutamate. Also, sufficient glutamate may be generated from ammonia assimilation via GS-GOGAT to make uptake of glutamate unnecessary under oligotrophic conditions. Serine, which like alanine has pyruvate as a deamination product, was also not favored as a growth substrate, which might reflect a consolidation of the supply of pyruvate through the uptake and catabolism of alanine, with serine directed toward other fates, such as C1 metabolism or glycine and threonine synthesis.

AlaDH and ammonia assimilation

In addition to being an important catabolic substrate, alanine is also the product of one of two major ammonia assimilation pathways in *S. alaskensis*.

Table 4 Comparison of genomic characteristics for five marine bacterial strains, highlighting disparate metabolic strategies

Features	*Sphingopyxis alaskensis RB2256*	*'Cand. Pelagibacter ubique' HTCC1062*	*Silicibacter pomeroyi DSS-3*	*Photobacterium angustum S14*	*Pseudoalteromonas haloplanktis TAC125*
Genome size (Mbp)	3.35	1.31	4.60	5.10	3.85
rRNA copy	1	1	3	12	9
Glycolysis genes	Entner–Doudoroff	Entner–Doudoroff (modified/incomplete)	Entner–Doudoroff	Embden–Meyerhof	Embden–Meyerhof
TCA cycle genes	Present	Present	Present	Present	Present
PHA synthesis genes	Present	Absent	Present	Present	Absent
ABC transport systems genes	7	12	58	33	5
Including amino acids	1	3	16	5	—
Polyamines	1	1	6	2	—
Sugars	2	2	8	2	—
Peptides	—	—	10	5	1
TRAP transport system genes	—	2	26	1	—
PTS transport genes	Absent	Absent	Present	Present	Absent
Transcription regulator families	AraC, LysR, LacI, GntR, TetR, Lrp, MarR, MerR, Fur	LysR, Fur	AraC, LysR, DeoR, LacI, GntR, TetR, Lrp, LuxR, MarR, MerR, Fur	AraC, LysR, DeoR, LacI, GntR, TetR, Lrp,LuxR, MarR, MerR	AraC, LysR, DeoR, LacI, GntR, TetR, Lrp, LuxR, MarR
Nitrate regulation genes	Present	NtrX/NtrY only	Present	Present	Present
Nitrate fixation genes	Present	Absent	Present	Present	Absent
Motility genes	Present	Absent	Present	Present	Present
Chemotaxis genes	Present	Absent	Absent	Present	Present
MCP genes	3	—	—	17	14

We present several lines of evidence that AlaDH is a major ammonia assimilatory enzyme in *S. alaskensis*. In most bacteria, the sequential action of GS and GOGAT serves as either the sole pathway for ammonia assimilation, or together with GDH (Kondorosi *et al.*, 1977; Westby *et al.*, 1987; Brown and Herbert, 1977a, b; Reitzer, 2003; Muro-Pastor *et al.*, 2005; Li and Lu, 2007). However, AlaDH plays a role in ammonia assimilation in some bacteria that have low or absent GDH activities. In these cases, GS-GOGAT remains the principal mechanism for ammonia assimilation, and AlaDH is used as an alternative or auxiliary ammonia enzyme under certain conditions, such as excess ammonia, when GS activity is repressed (Aharonowitz and Friedrich, 1980); inactivation of GS by MSO (Herbert *et al.*, 1978; Madigan and Cox, 1982; Caballero *et al.*, 1989a); or high pyruvate levels (Johansson and Gest, 1976; Moreno-Vivián *et al.*, 1983; Caballero *et al.*, 1989a). By contrast, in *S. alaskensis*, AlaDH activity was constitutive, and AlaDH seems to play a role in ammonia assimilation during growth across a range of ammonia concentrations and in the presence of various carbon sources (Table 3). AlaDH requires a different carbon skeleton to GDH and GS-GOGAT, and (like GDH) does not require ATP. Therefore, employing AlaDH in preference to GDH as an alternative assimilatory enzyme allows ammonia assimilation to proceed in the absence of a ready supply of 2-oxoglutarate. Thus, the selection of GS-GOGAT and AlaDH as the two major mechanisms for ammonia assimilation might be important under conditions of carbon limitation if the intracellular replenishment of 2-oxoglutarate is limited, such as during growth on substrates that are processed by the glyoxylate bypass (for example, PHA, acetate).

The fate of alanine in *S. alaskensis* seems to differ from that of other bacteria in which AlaDH is used to assimilate ammonia taken up by the cell. Phototrophic bacteria that use AlaDH as an assimilatory enzyme can convert alanine to glutamate, either directly using AlaAT (Johansson and Gest, 1976; Caballero *et al.*, 1989b) or through a series of transamination reactions (Herbert *et al.*, 1978; Cárdenas *et al.*, 1987). However, neither route seems to be available to *S. alaskensis*. Some of the accumulated alanine in *S. alaskensis* could be drawn off for biosynthetic purposes (for example, peptidoglycan, CoA, biotin, alanyl-tRNA), but the remainder cannot be directly converted to glutamate for further nitrogen metabolism. In the absence of suitable transaminases (for example, AlaAT) and a dedicated alanine deamination mechanism (Dad system), only AlaDH is available to catabolize intracellular alanine that is imported or generated endogenously. This again may constitute an example of metabolic simplification, by directing alanine away from nitrogen metabolism by way of glutamate or glutamine (Figure 1).

Oligotrophic lifestyle and trophic strategy of S. alaskensis

The relatively slow doubling time for *S. alaskensis* compared with specialist copiotrophs may be attributable to the loss of certain non-essential metabolic functions (for example, glutamate/alanine transamination; Dad system; 6-phosphogluconolactonase) (Figure 1); it has been shown earlier that the relatively slow growth rate ($<0.2\,h^{-1}$) is not the result of insufficient translational capacity (Fegatella *et al.*, 1998). A buffering of maximum growth rate might also relate to a metabolic emphasis on the synthesis of storage material (PHA, intracellular polysaccharide) to maintain a steady rate of cell division throughout periods of relative nutrient surfeit.

In addition to the ability of *S. alaskensis* to take up mixed substrates simultaneously (Schut *et al.*, 1995, 1997a), aspects of the carbon and nitrogen metabolism outlined above seem to be specifically geared toward an oligotrophic ecology, such as simplification of amino acid catabolism and alternate routes for ammonia assimilation that require different precursors, with the latter facilitating the efficient scavenging of ammonia under carbon-limited conditions (Figure 1). Overall, our data indicate an adaptive ecological strategy that is distinctive among marine bacteria, and adds to the repertoire of strategies so far ascribed to bacteria that survive and grow in the oceanic environment (for example, Moran *et al.*, 2004; Médigue *et al.*, 2005; Giovannoni *et al.*, 2005; Stocker *et al.*, 2008). The metabolic simplification of *S. alaskensis* is interpreted as an adaptation to conditions of nutrient depletion, especially carbon limitation, by focusing the uptake machinery on a narrow subset of bioavailable substrates, and ensuring that all aspects of metabolism are furnished by these substrates. This obviates the need for extensive gene regulation governing central carbon and nitrogen metabolism, especially regarding the fate of individual amino acids.

Nevertheless, our genomic analysis of *S. alaskensis* genome shows an array of regulatory genes, absent from SAR11. For *S. alaskensis*, this may reflect a capacity to alter metabolic strategies in response to ambient changes in the identity and concentration of both carbon and nitrogen substrates, such as in response to elevated nutrient levels (Table 4). Gene regulation is integral to an opportunistic strategy of efficiently exploiting competing substrates, whereas minimization of regulatory networks is one of the hallmarks of passive oligotrophs with streamlined genomes (Dufresne *et al.*, 2003; Giovannoni *et al.*, 2005). The small genome of '*Cand.* P. ubique' lacks genes associated with nutrient detection, motility, and most regulatory mechanisms (Table 4; Giovannoni *et al.*, 2005). The regulatory capacity of *S. alaskensis* is well illustrated by the expression of specific regulatory proteins associated with the Ntr and PtsNOP regulatory networks under nutrient-enriched conditions (Table 1), and the different responses of enzymes involved in ammonia assimilation to individual carbon and nitrogen sources (Table 3). The fact that *S. alaskensis* is flagellated (and many of the flagella and regulatory proteins associated with motility were detected by proteomics; Table 1) suggests that chemotactic ability provides a selective advantage in bulk low-nutrient waters. However, the possession of only a limited number of methyl-accepting chemotaxis proteins (MCPs) suggests that *S. alaskensis* has less scope to respond to diverse nutrient signals than copiotrophic bacteria.

The potential to detect, associate with and efficiently exploit elevated levels of substrates through a chemotactic response would distinguish *S. alaskensis* from passive oligotrophs (Polz *et al.*, 2006; Stocker *et al.*, 2008). The latter include SAR11 bacteria, for which the data indicate an oligotrophic strategy that entails obligate low-nutrient growth, low population densities, and a greater dependence on exogenous substrates to fulfill its metabolic requirements (Joint, 2008; Tripp *et al.*, 2008). The ubiquity of SAR11 bacteria suggests that this bacterial group may be specialized for extreme low-nutrient environments that are exposed to minimal nutrient fluxes (such as the Sargasso Sea) without compromising their ability to survive in large numbers in nutrient-enriched environments (Giovannoni *et al.*, 1990, 2005; Morris *et al.*, 2002). In contrast to SAR11 bacteria, *S. alaskensis* is metabolically poised to exploit high concentrations of substrates (amino acids, polyamines, carbohydrates). In support of this, it has been shown earlier for *S. alaskensis* that after nutrient upshift, starved or nutrient-limited chemostat cultures (from a range of carbon sources), resume maximum growth rates without a detectable lag (Fegatella and Cavicchioli, 2000; Cavicchioli *et al.*, 2003). However, *S. alaskensis* is not specialized for a strategy that entails rapid detection and exploitation of nutrient plumes or pulses, thereby setting it apart from specialist 'feast-and-famine' copiotrophs. Our genomic and experimental data for *S. alaskensis* are consistent with an ecophysiological trade-off between intense genomic streamlining and the ability to efficiently exploit fluxes in the quality and quantity of available nutrients.

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