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ORIGINAL ARTICLE

Transcriptome of the quorum-sensing signal-degrading *Rhodococcus erythropolis* responds differentially to virulent and avirulent *Pectobacterium atrosepticum*

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Social bacteria use chemical communication to coordinate and synchronize gene expression via the quorum-sensing (QS) regulatory pathway. In *Pectobacterium*, a causative agent of the blackleg and soft-rot diseases on potato plants and tubers, expression of the virulence factors is collectively controlled by the QS-signals *N*-acylhomoserine lactones (NAHLs). Several soil bacteria, such as the actinobacterium *Rhodococcus erythropolis*, are able to degrade NAHLs, hence quench the chemical communication and virulence of *Pectobacterium*. Here, next-generation sequencing was used to investigate structural and functional genomics of the NAHL-degrading *R. erythropolis* strain R138. The *R. erythropolis* R138 genome (6.7 Mbp) contained a single circular chromosome, one linear (250 kbp) and one circular (84 kbp) plasmid. Growth of *R. erythropolis* and *P. atrosepticum* was not altered in mixed-cultures as compared with monocultures on potato tuber slices. HiSeq-transcriptomics revealed that no *R. erythropolis* genes were differentially expressed when *R. erythropolis* was cultivated in the presence vs absence of the avirulent *P. atrosepticum* mutant *expI*, which is defective for QS-signal synthesis. By contrast 50 genes (< 1% of the *R. erythropolis* genome) were differentially expressed when *R. erythropolis* was cultivated in the presence vs absence of the NAHL-producing virulent *P. atrosepticum*. Among them, quantitative real-time reverse-transcriptase–PCR confirmed that the expression of some alkyl-sulfatase genes decreased in the presence of a virulent *P. atrosepticum*, as well as deprivation of organic sulfur such as methionine, which is a key precursor in the synthesis of NAHL by *P. atrosepticum*.
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INTRODUCTION

The plant microbiome encompasses up to thousands of microbial species which interact with each other and with their host, hence affecting health and reproduction of plants, as well as the productivity of crops (Mendes *et al.*, 2013; Berg *et al.*, 2014). Within microbiome, several bacterial species exhibit capacity to coordinate and synchronize gene expression at a population level using diffusible signals. The production, exchange and perception of these signals by bacteria define a gene-regulatory pathway called quorum-sensing (QS; Fuqua *et al.*, 1994). The QS-regulated functions are diverse and have crucial role in microbe–microbe and host-microbe interactions as they are involved in the production of antimicrobial compounds, expression of symbiotic and virulence functions, horizontal gene transfer, as well as formation of biofilms (Boyer and Wisniewski-Dyé, 2009). The plant microbiome also encompasses QS signal-degrading species which may interfere with QS-communication, hence quench the QS-regulated functions of the QS signal-producing species (d'Angelo-Picard *et al.*, 2005; Cirou *et al.*, 2007). Consequently, the interaction between QS signal-producing and -degrading species may directly affect microbiome dynamics and functions, and plant-host health. This paradigm is exemplified by the three-partner interaction involving the QS signal-degrading species *Rhodococcus erythropolis*, the QS signal-producing species *Pectobacterium atrosepticum* and their potato plant-host *Solanum tuberosum*.

P. atrosepticum is a causing agent of the blackleg and soft-rot diseases on potato plants and tubers (Toth *et al.*, 2011). These pathogenic bacteria produce and exchange, *N*-acylhomoserine lactones (NAHLs) as QS-signal molecules for controlling and coordinating expression of the virulence factors (Jones *et al.*, 1993; Pirhonen *et al.*, 1993). *Pectobacterium* mutants, which are defective or attenuated in the synthesis, accumulation or perception of the NAHL signals, are unable to express virulence factors and unable to induce disease symptoms on host plants (Pirhonen *et al.*, 1993; Smadja *et al.*, 2004; Liu *et al.*, 2008; Crépin *et al.*, 2012a). Transcriptomics revealed that QS controls expression of 1100 genes in *Pectobacterium*; hence up to a quarter of its genome, including most of the virulence factors, the plant cell-wall degrading enzymes and their secretion systems, as well as several metabolic pathways (Liu *et al.*, 2008). Because of the key role of QS in virulence, NAHL signals are putative targets for developing anti-virulence strategies against *Pectobacterium*-induced symptoms (Dong *et al.*, 2007; Faure and Dessaux, 2007; Crépin *et al.*, 2012b). One of these strategies uses particular bacterial strains as biocontrol

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agents that are able to cleave and degrade NAHL and disrupt QS-signaling of the plant pathogen *Pectobacterium*.

NAHL-degrading bacterial strains belong to different phyla such as Proteobacteria (e.g., *Agrobacterium*, *Ralstonia*, *Ochrobactrum*), Firmicutes (*Bacillus*) and Actinobacteria (*Rhodococcus*). In the plant pathogen *Agrobacterium tumefaciens*, two cytoplasmic lactonases were shown to be involved in opening of the gamma-butyrolactone ring of NAHLs, and their expression was controlled by plant-derived compounds (Zhang *et al.*, 2002; Carlier *et al.*, 2003; Chevrot *et al.*, 2006; Haudecoeur *et al.*, 2009a, b). *R. erythropolis*, which is a common inhabitant of the potato plant environment (Cirou *et al.*, 2011; Diallo *et al.*, 2011), efficiently degrades NAHL signals through three intracellular enzymatic activities: lactonase, amidase and reductase (Uroz *et al.*, 2009). The reductase alters ketone function on acyl chain of the NAHL molecules, whereas the lactonase and amidase hydrolyze NAHLs to by products that are proposed to be assimilated through fatty acid oxidation pathways. Regulation of their expression by NAHL or other signals is unknown. Because NAHL molecules diffuse through cell membranes, *R. erythropolis* is able to degrade NAHL, even its NAHL-degrading enzymes are not secreted. Only the lactonase-encoding gene *qsdA* was identified in *R. erythropolis* (Uroz *et al.*, 2008), whereas the genetic determinants coding for the reductase and amidase activities are still unknown. QsdA was showed to be involved in the cleavage of NAHLs and related lactones, such as gamma-heptanolactone and gamma-hexanolactone, which are assimilated as a sole carbon and energy source by *R. erythropolis* (Uroz *et al.*, 2008; Barbey *et al.*, 2012; Cirou *et al.*, 2012). Because of its ability to cleave NAHLs, *R. erythropolis* is proposed as a plant-protecting and anti-biofouling agent (Cirou *et al.*, 2007; Faure and Dessaux, 2007; Oh *et al.*, 2012). In addition to NAHL catabolism, *R. erythropolis* exhibits the capability for degrading hydrocarbons and xenobiotics, hence this bacterium is used as a bioremediation agent for the treatment of polluted soils and waters (de Carvalho and da Fonseca, 2005). Particularly, *R. erythropolis* may exhibit the capability to cleave C-S bonds in fossil-fuel compounds using four desulfurization enzymes (Ohshiro *et al.*, 1997). Because of the structure of NAHL, consisting in a homoserine lactone linked to an acyl chain, involvement of fatty acid metabolism in NAHL degradation is expected.

Although the capacity of the NAHL-degrading *R. erythropolis* to obliterate QS-pathway, hence to markedly reduce the virulence gene expression in *P. atrosepticum* is well documented (Cirou *et al.*, 2007; Barbey *et al.*, 2013), how *P. atrosepticum* could modify the *R. erythropolis* transcriptome is not known. In this work, we reported the structural and functional genomics of the NAHL-degrading *R. erythropolis* strain R138. Specifically, we compared the *R. erythropolis* transcriptomes when it colonized tuber slices in the presence of either the potato pathogen *P. atrosepticum* CFBP6276 or its avirulent derivative that is defective for the synthesis of NAHL signals. This work reported a first transcriptome of a NAHL-degrading bacterium in the presence of a NAHL-producing one.

MATERIALS AND METHODS

Bacterial strains

R. erythropolis R138 (Cirou *et al.*, 2007) and *P. atrosepticum* CFBP6276 (Kwasiborski *et al.*, 2013), as well as its *expI* Km^R-derivative, which is defective for producing the NAHL signals and virulence factors (Latour *et al.*, 2007), were routinely cultivated at 28 °C in TY medium (0.5% tryptone, 0.3% yeast extract) supplemented with kanamycin (50 μg ml^{-1}) when appropriate. The NAHL-biosensor *Agrobacterium tumefaciens* NT1(pZLR4) was cultivated in *Agrobacterium* Broth (AB) medium supplemented with mannitol (2 g l^{-1}), NH_4Cl (1 g l^{-1}) and gentamycin (25 μg ml^{-1}) as described previously (Cha *et al.*, 1998).

Culture conditions with inorganic and organic sulfur sources

Cultures of *R. erythropolis* R138 in liquid AB medium with gamma caprolactone (2 g l^{-1}) as a carbon source were washed twice in 0.8% NaCl. Bacteria were inoculated at OD_{600} value of 0.6 in fresh AB medium supplemented with inorganic ($MgSO_4$) and organic (methionine) sulfur sources at 24 and 0.24 mM. After 6 h of incubation at 30 °C, one volume of RNA protect Cell Reagent (Qiagen, Hilden, Germany) was added to the culture and was frozen at − 80 °C until use. Each of the four tested culture conditions (two sulfur sources at two concentrations) was replicated three times.

Genomics

Total DNA of *R. erythropolis* R138 was extracted from the pellet of a bacterial suspension adjusted to an optical density of 0.6 at 600 nm. The cell lysis was carried out at 37 °C during 2 h in 0.5 ml of TES buffer pH 8 (10 mM Tris, 1 mM EDTA and 150 mM NaCl) supplemented with 20 mg ml^{-1} lysozyme (Eurobio, Les Ulis, France) and 133 μg ml^{-1} RNase (Sigma-Aldrich, St-Louis, MO, USA). Then, 100 μl of 20% w/v SDS were added before gentle mixing. One volume of phenol/choroform/isoamylic acid (25:24:1, Sigma-Aldrich) was added, mixed and centrifuged for 15 min at 15 000 *g*. The aqueous phase was washed twice by addition of one volume of Aquaphenol (MP bio, Santa Ana, California, USA). The genomic DNA was precipitated by one volume of isopropanol. After a centrifugation for 10 min at 15 000 *g*, the DNA was washed by an addition of a volume of 70% ethanol followed by centrifugation for 10 min at 15 000 *g*. The pellet was dried before the DNA was solubilized in TE buffer pH 7.5 (10 mM Tris-HCl, 1 mM EDTA). The DNA concentration was estimated using the Nanodrop 1000 spectrophotometer (Thermo Scientific, Whaltham, MA, USA) and frozen until used.

De novo genome assembly of *R. erythropolis* R138 was performed by combining Illumina and 454-Roche technologies as described by Kwasiborski *et al.* (2014). Reads were collected from three genomic libraries: two short-fragment libraries (300 bp and 380 bp) used for paired-end 2 × 72 Illumina-sequencing (ImaGif, Paris, France) and single read 454-sequencing (Eurofins MWG, Ebersberg, Germany), and a long-paired end library with an insert size of 8 kbp used for 454-sequencing (Eurofins MWG). Assembly was carried out using the CLC Genomics Workbench v5.1 (CLC bio, Aarhus, Denmark) with read length at 0.5 and similarity at 0.8 as parameters. The coding sequences and their functions of the genome were predicted using the Rapid Annotation using Subsystem Technology v4.0 (RAST) automated pipeline (Aziz *et al.*, 2008). The genome of *R. erythropolis* R138 was deposited at NCBI (http://www.ncbi.nlm.nih.gov/) under the GenBank reference ASKF00000000.

Plant assay

Inoculation of bacteria on potato tuber slices was adapted from a protocol previously described by Kwasiborski *et al.*, (2012). Bacteria cultivated in TY medium were washed in saline water (0.8% w/v NaCl) and the cell number was adjusted to 10^9 c.f.u. ml^{-1}. Tuber slices (1 cm) of *Solanum tuberosum* variety Allians were covered with a membrane (Supor 450, diameter 25 mm, pore size of 0.45 μm, PALL, NY, USA) and inoculated by 300 μl of three cell suspensions: *R. erythropolis* R138 alone, a mix (ratio 10:1) between *R. erythropolis* R138 and *P. atrosepticum* CFBP6276, and a mix (ratio 10:1) between *R. erythropolis* R138 and the *P. atrosepticum expI* mutant. The 10:1 ratio between *R. erythropolis* and *P. atrosepticum* allowed a reduction of symptoms on potato tuber, as described previously (Cirou *et al.*, 2007). The population dynamics of *R. erythropolis* and *P. atrosepticum* on potato tuber slices were measured every 3 hours over a period of 27 h. Bacteria on membranes were recovered by vortexing in saline water (2 ml). Serial dilutions of the bacterial suspensions were plated on TY medium supplemented with X-gal (40 μg ml^{-1}) and incubated at 25 °C for 4 days before counting. *R. erythropolis* R138 appeared as white colonies, whereas *P. atrosepticum* CFBP6276 and its *expI* derivative appeared as blue colonies. Experiments were performed in triplicates.

NAHL extraction and quantification

NAHL extraction and quantification procedures were adapted from Cha *et al.* (1998) Briefly, bacterial cell suspensions recovered from potato slices, were centrifuged for 10 min at 15 000 *g*. NAHLs were extracted from the supernatant by addition of one volume of ethyl acetate and air-drying the organic fraction. The extracted NAHLs were dissolved in 20 µl of ethyl acetate, of which 5 µl was spotted on TLC (thin layer chromatography) silica plates (Macherey-Nagel, Düren, Germany). TLCs were overlaid with the NAHL-biosensor strain *A. tumefaciens* NT1(pZLR4) in AB medium supplemented with agar (15 g l^{-1}) and X-gal (40 µg ml^{-1}). For the quantification of NAHLs, a calibration curve was obtained with pure NAHL, 3-oxo-octanoylhomoserine lactone (Sigma-Aldrich).

Transcriptomics

After 22-hour incubation of potato slices at 25 °C, bacteria were collected from membranes by vortexing in 1 ml of 50% RNAprotect Cell Reagent. After centrifugation for 10 min at 20 800 *g*, bacterial pellets were frozen at − 80 °C until use. Each of the three conditions, *R. erythropolis* R138 alone and *R. erythropolis* R138 in association with either *P. atrosepticum* CFBP6276 or its *expI* derivative were replicated three times. The total RNA extraction was carried out using the NucleoSpin RNA II (Macherey-Nagel) according to the manufacturer's recommendations. The RNAseq analysis was performed using ProfileXpert (University of Lyon I, Lyon, France). RNA control was carried out using the 2100 Bioanalyzer Instrument (Agilent, Santa Clara, CA, USA), then Ribo-Zero rRNA Removal Meta-bacteria kits (tebu-bio, Le Perray en Yvelines, France) was used for rRNA depletion. Directional RNA paired-end libraries were sequenced (2 × 50 bp) on Illumina Hiseq-2500. The number of aligned reads per predicted gene were counted and normalized according to the gene length to calculate for each gene the number of reads per Kilo base per Million mapped reads (RPKM). No interspecies hybridization was observed between the genome of the actinobacterium *R. erythropolis* R138 and that of the proteobacterium *P. atrosepticum* CFBP6276 (Kwasiborski *et al.*, 2013).

The differentially expressed genes of *R. erythropolis* R138 in the presence vs absence of *P. atrosepticum* CFBP6276 and its *expI* mutant were identified using DEseq v1.9.6, an R program language package available through the Bioconductor platform (http://www.bioconductor.org/packages/release/bioc/html/DESeq.html). DEseq estimates the variance-mean dependence in count data from high-throughput sequencing assays and tests for differential expression based on a model using the negative binomial distribution (Anders and Huber, 2010). We also listed the highly expressed genes of *R. erythropolis* when it colonized the potato tuber slices alone. We considered only genes exhibiting a RPKM median value, which was over by three times the RPKM median value of all genes. All predicted genes of which the standard deviation of RPKM was over the RPKM mean value were discarded of the analysis.

Quantitative real-time reverse-transcriptase–PCR (qRT-PCR)

Expression of several genes was quantified by qRT-PCR using biological triplicates. Sequences and characteristics of used primers are presented in the Supplementary Table S1. Reverse transcriptions were carried out using the protocol for high GC content bacteria from the Revert Aid Reverse Transcriptase according to the manufacturer's recommendations (Fermentas, Whaltham, MA, USA). A Light Cycler 480 (Roche Applied Science, Penzberg, Germany) and Light Cycler 480 SYBR Green I Master (Roche Applied Science) were used for quantitative PCR. The 15 µl final-volume mix contained SYBR Green I Master (1x), forward and reverse primers (1 µM) and 0.01 µg of cDNA samples. After denaturation at 95 °C for 10 min, the amplification and quantification program was repeated 45 times as follows: 95 °C for 15 s, 60 °C for 15 s, 72 °C for 20 s, with a single fluorescence measurement, followed by the melting curve program (65 °C–95 °C with a heating rate of 0.1 °C s^{-1} and a continuous fluorescence measurement) and a final cooling step at 45 °C. The recombinase A (*recA*) gene was used as a reference gene.

RESULTS

Genome architecture of *R. erythropolis* R138

The genome of *R. erythropolis* R138 consisted of one circular chromosome (6 444 726 bp), a linear plasmid (247 675 bp) and a circular plasmid (84 151 bp). The G+C content was homogenous among the replicons, from 60–62% (Figure 1). The chromosome sequence resulted from the assembly of nine contigs from 5.5 kbp to 2.7 Mbp, which were spaced out by undetermined sequences measuring from 10 to 5500 bp. The linear plasmid sequence was composed of two contigs (22 and 220 kbp), which were separated by a single gap measuring ~ 3.8 kbp. Finally, the circular plasmid sequence resulted from a single contig of 84 kbp. All characteristics of contigs and gaps are indicated in Supplementary Table S2. Using the GC skew calculation (Perna and Kocher, 1995), replication origins could be located on the chromosome and the circular plasmid and were used for starting the nucleotide numbering. Using the automated annotation RAST software (Table 1), 6562 open reading frames (ORFs) were predicted on the reverse and forward strands (Figure 1). Twelve *rRNAs* and 50 tRNAs were identified. From 86–94% of *R. erythropolis* R138 genome sequence in length was shared by genomes of the *R. erythropolis* strains isolated from different environments: PR4 from ocean, XP and DN1 from oil-contaminated

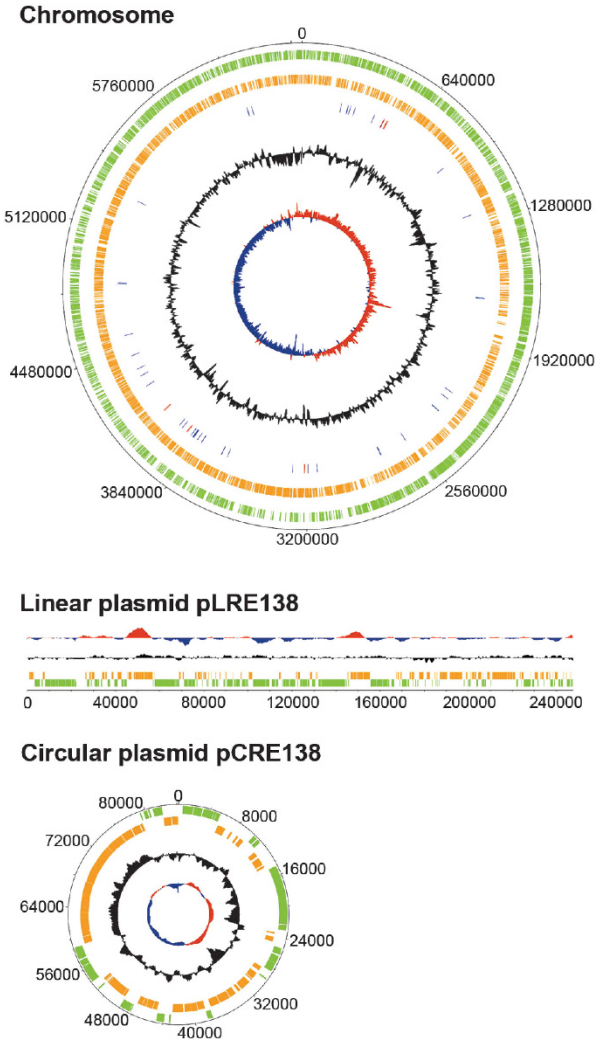

Figure 1 Map of the *R. erythropolis* R138 genome. The innermost and second circles highlight GC skew and GC content (%), respectively. The third circle shows RNA genes location (tRNAs in blue color, rRNAs in red, other RNAs in black). The fourth and fifth circles show the distribution of genes on the reverse (orange) and forward (green) strand, respectively.

Table 1 Number of genes per RAST-functional categories in the genome (columns 2 and 3) and transcriptome (columns 4 and 5) of *R. erythropolis* R138

RAST-functional categories	*Genome*		*Transcriptome on tuber*	
	No of total ORF	*Percentage of total ORF*	*No of expressed ORF on potato*	*Percentage of total expressed ORF*
Amino acid metabolism	361	5.5	5	2.7
Carbohydrate metabolism	351	5.3	8	4.3
Cell division and cell cycle	21	0.3	2	1.1
Cell wall and capsule	69	1.1	3	1.6
Cofactors, vitamins and prosthetic pigments	287	4.4	4	2.1
DNA metabolism	85	1.3	2	1.1
Dormancy and sporulation	4	0.1	0	0.0
Fatty acids. lipids and isoprenoids metabolism	268	4.1	5	2.7
Iron metabolism	21	0.3	0	0.0
Membrane transport	87	1.3	3	1.6
Aromatic compounds metabolism	72	1.1	0	0.0
Motility and chemotaxis	10	0.2	0	0.0
Nitrogen metabolism	33	0.5	0	0.0
Nucleosides and nucleotides	103	1.6	3	1.6
Phosphorus metabolism	27	0.4	0	0.0
Potassium metabolism	13	0.2	0	0.0
Protein metabolism	211	3.2	6	3.2
Regulation and cell signaling	58	0.9	2	1.1
Regulons	5	0.1	0	0.0
Respiration	98	1.5	0	0.0
RNA metabolism	89	1.4	5	2.7
Secondary metabolism	8	0.1	0	0.0
Stress response	107	1.6	3	1.6
Sulfur metabolism	75	1.1	3	1.6
Virulence, disease and defense	113	1.7	1	0.5
NA	3921	59.7	132	70.6
RNA	65	1.0	0	0
Classified ORF	2576	39.3	55	29.4
Total ORF	6562	100.0	187	100.0

Abbreviations: NA, not applicable; No, number of; ORF, open reading frame; RAST, Rapid Annotation using Subsystem Technology v4.0; RNA, ribonucleic acid.

soil, SK121 from human skin and CCM2595 from soil (Sekine *et al.*, 2006; Vesely *et al.*, 2007; Shevtsov *et al.*, 2013). By contrast, number, size and functions of plasmids differed when the *R. erythropolis* strains were compared.

Among the predicted *R. erythropolis* R138 ORFs, only 2576 (39.3%) were classified in the RAST-functional categories, showing that the protein annotation was poorly documented in this species. Among them, five categories (metabolism of amino acids, carbohydrates, cofactors, proteins and fatty acids) were the most represented in the genome of *R. erythropolis* R138. Fatty acid and sulfur metabolism are the most studied pathways in *R. erythropolis* (de Carvalho and da Fonseca, 2005). Interestingly, in the strain R138, 75 and 268 ORFs are involved in the metabolisms of sulfur and fatty acid, respectively. Especially, the chromosome and linear plasmid harbored a large variety of desulfurating enzymes: five dibenzothiophene desulfurization enzymes that break thioether bonds and six alkylsulfatases, and five arylsulfatases that remove sulfate from diverse carbon skeletons. *R. erythropolis* R138 also possessed a high number of genes related to acyl-chain degradation and recycling: as an example, the family of long-chain fatty acid-CoA ligases, which are involved in activation of the fatty acids in the course of beta-oxidation, is represented by 66 ORFs.

R. erythropolis R138 and *P. atrosepticum* CFBP6276 co-exist on potato tubers

On potato slices, *R. erythropolis* R138 and *P. atrosepticum* CFBP6276 (or its *expI* derivative) grew alone and in mixed populations (Figure 2a), confirming that *R. erythropolis* R138 did not express antibiotic activities against *P. atrosepticum* CFBP6276 (Uroz *et al.*, 2008). From the inoculation time to the stationary phase (20 h later), the bacterial populations increased by three log. Interestingly, when *P. atrosepticum* CFBP6276 grew alone, NAHL accumulated up to 6 pmol per membrane, whereas in the presence of the NAHL-degrading strain *R. erythropolis* R138, NAHL only reached up to 7 fmol per membrane (Figure 2b). As a consequence, NAHL-dependent maceration of the potato tissues decreased dramatically in the presence of the plant-protective *R. erythropolis* R138 (Figure 2c). Hence, the experimental conditions were appropriate for analyzing transcriptomes of *R. erythropolis* in response to the presence of *P. atrosepticum* on potato tubers.

Transcriptome of *R. erythropolis* was not modified by a NAHL-defective *P. atrosepticum*

No differentially expressed genes were identified when transcriptomes of *R. erythropolis* R138 which grew alone on potato tuber slices were compared with those in the presence of the *P. atrosepticum expI*

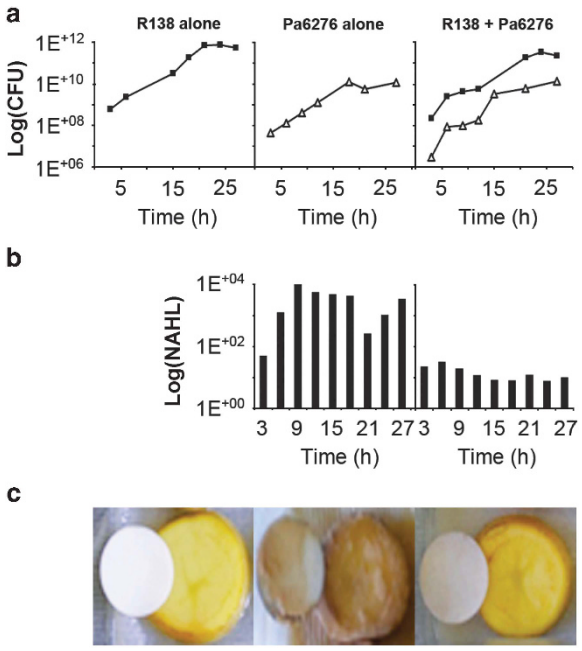

Figure 2 Colonization of potato tubers by *R. erythopolis* and *P. atrosepticum*. (**a**) Growth (c.f.u. per tuber slice) of *R. erythropolis* R138 (square) and *P. atrosepticum* CFBP6276 (triangle) populations colonizing the potato tuber slices; (**b**) Quantification of the NAHL signals (fmol per tuber slice) emitted by *P. atrosepticum* CFBP6276 (**c**); Symptoms observed 24 h after inoculation of *R. erythropolis* R138, *P. atrosepticum* CFBP6276 and a combination of both the populations on potato tuber slices. All assays for cell growth, NAHL and symptom monitoring were performed in triplicates.

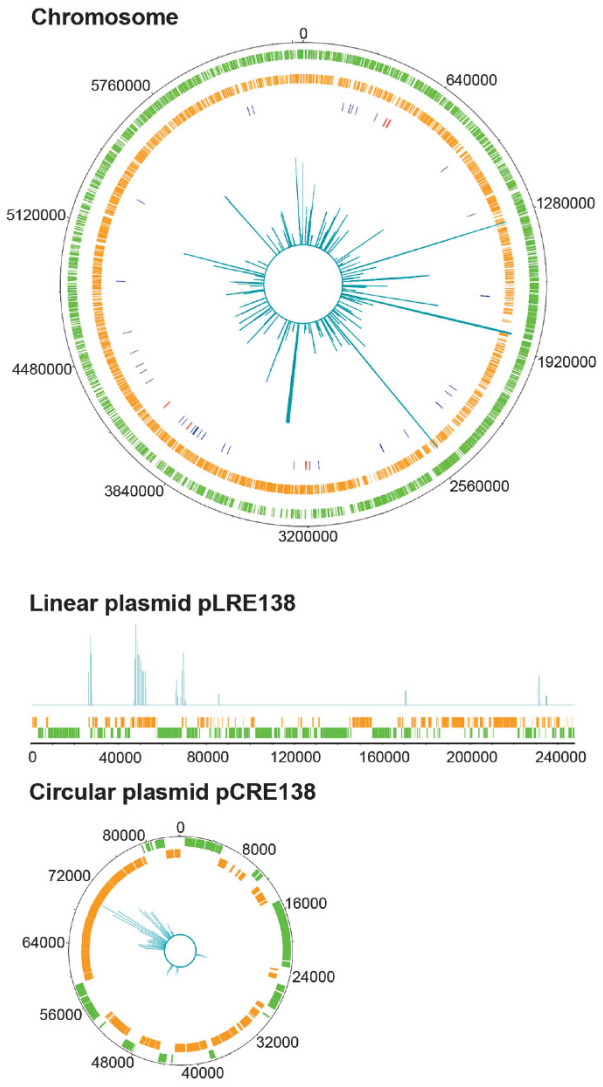

Figure 3 Highly expressed genes of *R. erythropolis* R138 colonizing the potato tubers. Distribution and relative expression level of the highly expressed genes when *R. erythropolis* R138 colonized alone the potato tuber slices. We showed only genes exhibiting a RPKM median value which was over by three times the RPKM median value of all genes. All these genes are listed in Supplementary Table S3. RNA extraction and sequencing were performed in triplicates.

mutant that is defective for NAHL synthesis. This experiment indicated that *R. erythropolis* did not modify its gene-expression profile in the presence of an avirulent pathogen. Under this condition, 187 genes were considered as highly expressed in *R. erythropolis* R138 (Supplementary Table S3). Genes coding for tRNA and rRNA were not considered in this list of unique genes. These highly expressed genes exhibited a homogeneous distribution along the chromosome whereas some gene clusters could be identified on the linear and circular plasmids (Figure 3). The functions of these expressed gene clusters on the plasmids were all unknown. Actually, a functional RAST-category could be assigned to only 29% (55 ORFs) of all the highly expressed genes (Table 1). Interestingly, several genes related to fatty acid and sulfur metabolisms, which are the most studied pathways in *R. erythropolis*, were highly expressed when *R. erythropolis* R138 colonized the potato tuber slices. They were 3-ketoacyl-CoA thiolases (ORF1420 and ORF4007), long-chain fatty acid-CoA ligase (ORF2117 and ORF3497) and phosphatidylserine decarboxylase (ORF1766), which all belong to the fatty acid metabolism, and alkylsulfatases (ORF4771 and ORF4816) and thiosulfate sulfur transferase (ORF4081) associated to sulfur metabolism. The identification of these highly expressed genes does not exclude that other genes, which are expressed at a lower level, are involved in potato tuber colonization.

R. erythropolis transcriptome is modified in response to the virulent *P. atrosepticum*

When the transcriptome of *R. erythropolis* R138 which grew alone on potato slices was compared with that of *R. erythropolis* R138 in the presence of the virulent *P. atrosepticum* CFBP6276, 50 genes were differently expressed: 22 and 31 were down and upregulated, respectively. The localization and expression levels of these genes, their functions according to the RAST annotation and fold change according to the DEseq analysis are given in the Supplementary Data (Supplementary Figure S1 and Supplementary Table S4). Among the downregulated genes, three hypothetical protein genes were localized on the linear plasmid. The others belonged to the chromosome. The downregulated genes were potentially implicated in cofactor metabolism (ORF2389, ORF3251, ORF5957 and ORF5958) and sulfur metabolism (ORF4771, ORF4816 and ORF4877) or exhibited unknown functions (15 ORFs). All the upregulated genes were localized on the chromosome. Twenty genes exhibited unknown functions, whereas the remaining 11 were associated with the metabolism of the carbohydrates (ORF4921), cofactors (ORF1288), DNA (ORF462), iron (ORF2860), transport across membrane

(ORF2124), nitrogen (ORF2140), nucleosides and nucleotides (ORF4620), regulation (ORF775), RNA (ORF539 and ORF1958) and response to stress (ORF1552). The *qsdA* gene (ORF5278) encoding NAHL-degrading lactonase was not differentially expressed, nor highly expressed under the analyzed experimental conditions. Moreover, no putative amidases or reductases were identified among these differentially expressed genes.

Expression of the *R. erythropolis* alkylsulfatases on potato tuber and in pure culture

Among the differentially expressed genes, the three alkylsulfatases (ORF4771, ORF4816 and ORF4877) retained our attention because their synchronized downregulation could reveal a common response to a particular environmental change, such as a variation in nutrient availability which would be induced by the presence of the virulent *P. atrosepticum* without any influence on the *R. erythropolis* R138 growth. Alkyl-sulfatases are expected to be implicated in the sulfur metabolism and/or recycling of sulfured lipid derivatives. They are of special interest in the studied three-partner interaction because (i) potato tuber is known to be a sulfur-limited environment (Subramanian *et al.*, 2011); (ii) sulfur pathways are especially developed in *R. erythropolis* species (de Carvalho and da Fonseca, 2005); and (iii) organic sulfur (methionine) is a key precursor of NAHL synthesis in *Pectobacterium* (Crépin *et al.*, 2012c). HiSeq-transcriptomic data showed that, in the absence of the virulent *P. atrosepticum* CFBP6276, RPKM values were high (between 4370 and 8300), whereas they strongly decreased in its presence (Figure 4a). This differential gene expression was confirmed by qRT-PCR on RNAs extracted from an independent experiment on newly infected potato tubers (Figure 4b). We also investigated whether level and type of the sulfur-source (organic and inorganic) could alter expression of these three alkyl-sulfatase-encoding genes. Although no variation was observed between high and low sulfate concentrations, the expression of the alkylsulfatases ORF4816 and ORF4877 decreased with the level of methionine (Figure 5).

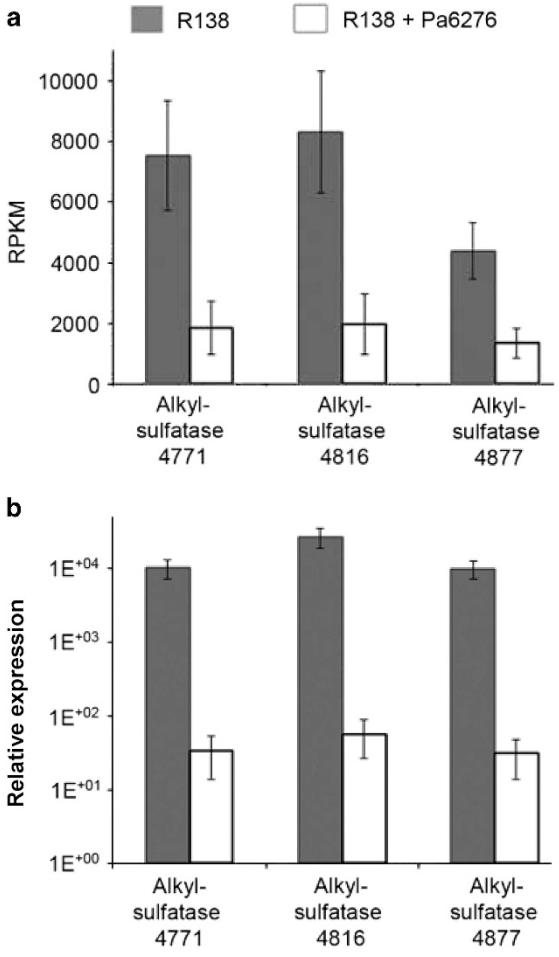

Figure 4 *In planta* expression of alkylsulfatases ORF4771, ORF4816 and ORF4877. Gene expression was measured using RNAseq (**a**) and qRT-PCR (**b**) when *R. erythropolis* R138 colonized alone the potato tuber slices (gray bars) and with *P. atrosepticum* CFBP6276 (white bars). Relative gene expression is normalized using that of *recA* gene. Experiments were performed with three biological triplicates.

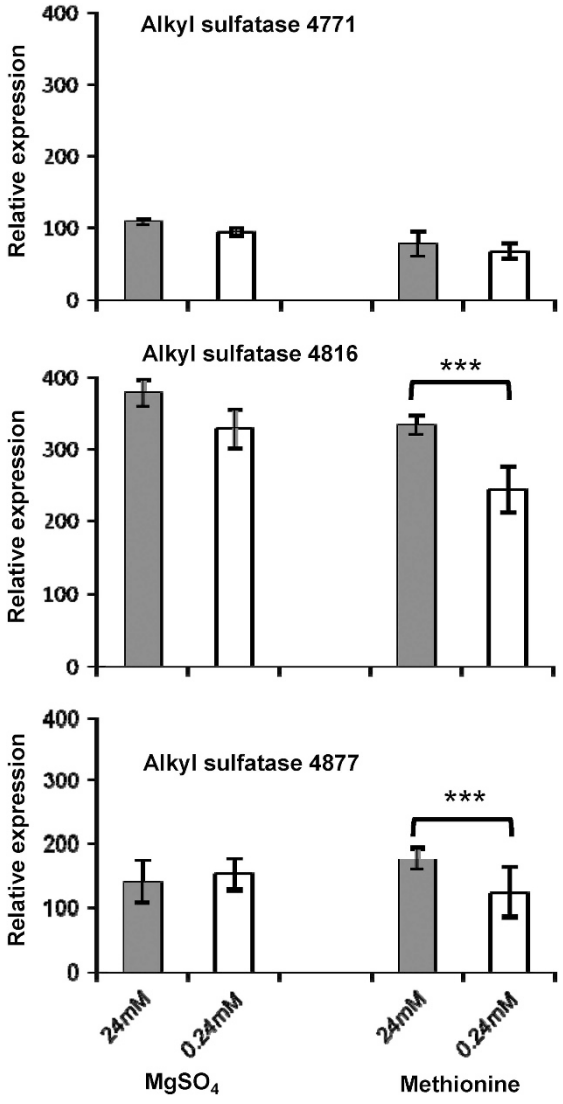

Figure 5 *In vitro* expression of alkylsulfatases ORF4771, ORF4816 and ORF4877. *R. erythropolis* R138 was cultivated in the presence of high (gray bars) and low (white bars) concentration of $MgSO_4$ or methionine. Relative gene expression is normalized using that of *recA* gene. Experiments were performed with three biological replicates. Statistical differences were assessed using the Mann–Whitney test ($P<0.05$).

DISCUSSION

HiSeq-transcriptomics revealed that the NAHL-degrading *R. erythropolis* responded differentially to virulent and avirulent *P. atrosepticum* when they were co-cultured on potato slices. Fifty genes were differentially expressed when *R. erythropolis* was cultivated in the presence vs absence of the NAHL-producing virulent *P. atrosepticum*. By contrast, no differentially expressed genes were identified when *R. erythropolis* was cultivated in the presence vs absence of an avirulent *P. atrosepticum* which is defective for NAHL production. These two different responses are discussed on the light of the NAHL-degrading capability of *R. erythropolis* and the environmental changes that virulent *P. atrosepticum* provokes on potato tubers.

When *R. erythropolis* and avirulent *P. atrosepticum* were co-cultured, growth curves and transcriptomics showed that the interactions between the two bacteria and especially the response of *R. erythropolis* were negligible or insignificant. This suggests that under our experimental conditions, the presence of the avirulent *P. atrosepticum* is neutral for *R. erythropolis*. This result also indicates that the wounded tuber slices as a growth environment provide enough nutrients to support multiplication of both species without apparent alteration of the plant tissues. This work exemplified a neutral kind of microbe–microbe interaction that is poorly analyzed in the literature. Most of the previous studies focused on competitive and syntrophic interactions between microbes. In these cases, when a microbe enhanced or inhibited the growth of a given bacterium, the transcriptomic analyzes highlighted a shift in the expression of up to hundreds of genes (i.e., more than 10% of the studied genomes) which are implicated in gene regulation, stress responses, metabolic functions and production of antimicrobial compounds (Johnson *et al.*, 2006; Maligoy *et al.*, 2008; Nouaille *et al.*, 2009; Garbeva *et al.*, 2011; Liu *et al.*, 2011). This variation in gene expression was proposed to be a consequence of the presence of other microbes and/or the alteration of the environment.

When *R. erythropolis* and virulent *P. atrosepticum* were co-cultured, the growth curves of both bacteria were largely comparable with those of the bacteria grown alone on the potato tubers. Nevertheless, the NAHL-controlled virulence functions were dramatically reduced in *P. atrosepticum*, strongly reducing the alteration of the plant tissues. Under these conditions, the differentially expressed genes in *R. erythropolis* represented $<1\%$ of its genome; 22 and 31 of them were down and upregulated, respectively. *R. erythropolis* expresses at least three NAHL-degrading activities that are reductase, amidase and lactonase, but only the lactonase-coding gene *qsdA* was identified (Uroz *et al.*, 2008 and 2009). Neither *qsdA* nor the genes encoding putative reductase, amidase and lactonase were present among the differentially expressed genes. In our assays, *qsdA* remained expressed at a low level in the presence or absence of *P. atrosepticum*. Recent work reported that a *R. erythropolis* R138 defective for *qsdA* was only weakly affected in its capacity to degrade NAHLs and limit the symptoms induced on tubers by *P. atrosepticum* (Barbey *et al.*, 2013). Thus even if QsdA contributes to NAHLs degradation, other enzymes are expected to have an important role.

When *R. erythropolis* colonized the potato tuber, a major part of highly expressed genes were related to primary metabolism, probably in order to sustain the observed growth. These genes are potentially involved in metabolism of sugars, amino acids, lipids and ribonucleotides, which are the compounds usually encountered in tubers (Jansky, 2010). Additional genes which exhibit a lower expression level were expected to be also involved in multiplication of *R. erythropolis* on potato tuber slices. A major characteristic of *R. erythropolis* species is their capability to assimilate a wide variety of carbon and sulfur sources from natural and xenobiotic compounds (de Carvalho and da Fonseca, 2005; Urai *et al.*, 2007; Aggarwal *et al.*, 2011). This metabolic versatility would contribute to the distribution of *R. erythropolis* in a wide variety of environments such as oceans and soils, including those polluted by fuels and pesticides. As with soil, potato tuber is considered as a sulfur-limited environment (Subramanian *et al.*, 2011). Noticeably, our results highlighted three alkyl-sulfatase genes (ORF4771, ORF4816 and ORF4877) of which the expression was downregulated in the presence of the plant pathogen *P. atrosepticum* CFBP6276. Because of their synchronized variation, the alkyl-sulfatase expression could be considered as a signature of the *R. erythropolis*-*P. atrosepticum* interaction in the potato environment. These enzymes are implicated in the cleavage of C-OS bond of ester sulfates to release acyl chain and sulfate and could contribute to the recycling and assimilation of carbon and sulfur sources (Kertesz, 2000; Hanson *et al.*, 2004; Sogi *et al.*, 2013). Additional assays revealed that expression of alkylsulfatases ORF4816 and ORF4877 decreased as the level of organic sulfur (methionine) decreased, suggesting that their expression depends on the availability of organic sulfur. We therefore speculate that the virulent pathogen depletes organic sulfur from the potato slices for its use as a precursor of NAHLs or other biomolecules, such as the secreted virulence proteins (plant cell-wall degrading enzymes). This work highlights a novel trait related to sulfur metabolism in a microbe–microbe interaction associating the NAHL-producing and NAHL-degrading bacteria.

When *P. atrosepticum* interacts with potato plants, two phases can be discerned: an asymptomatic colonization phase, which may occur along the vegetative development of the plant host, followed by a symptomatic phase, in which the blackleg and soft-rot diseases are expressed (Pirhonen *et al.*, 1993; Smadja *et al.*, 2004; Latour *et al.*, 2007; Liu *et al.*, 2008). On the basis of our results on the 3-partner interaction, we postulate a differential response of the biocontrol agent *R. erythropolis* during both phases of the interaction of *P. atrosepticum* on potato. Indeed, the *R. erythropolis* expression profile is not modified in the presence of the avirulent *P. atrosepticum expI* mutant, which may be considered as a mimic of the pathogen during the asymptomatic phase. In contrast, during the symptomatic phase, *R. erythropolis* differentially expresses a particular subset of genes and seems to experience some level of methionine deprivation, possibly as a consequence of NAHL synthesis by the virulent *P. atrosepticum*. Nevertheless, *R. erythropolis* inactivates part of these NAHLs limiting the disease development. As none of the differentially expressed genes putatively encodes any of the three known NAHL-degrading activities, it is currently unclear how *R. erythropolis* accomplishes NAHL inactivation. With the limited set of differential genes at hand, the road to address this enigma is wide open. As a conclusion, this work designates sulfur metabolism as a new way to be explored to improve our knowledge of the interaction between the plant pathogen *P. atrosepticum* and the NAHL-degrading *R. erythropolis*, as well as to enhance the protective effect of *R. erythropolis* as a biocontrol agent.

DATA ARCHIVING

Repository/DataBank Accession: Genbank
Accession ID: ASKF00000000
Databank URL: http://www.ncbi.nlm.nih.gov/nuccore/ASKF00000000

Repository/DataBank Accession: Genbank
Accession ID: ASAB00000000
Databank URL: http://www.ncbi.nlm.nih.gov/nuccore/ASAB00000000.1

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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