Pathogen reservoirs

Chironomid egg masses and *Vibrio cholerae*

Cholera is a severe diarrhoeal disease triggered by a toxin produced by specific biotypes of the bacterium *Vibrio cholerae* that are pathogenic only to humans. There seems to be no chronic state of the disease and the natural reservoir of the pathogen is environmental[1]. Here we show that egg masses of the non-biting midge *Chironomus* sp. (Diptera) harbour *V. cholerae* and act as its sole carbon source, thereby providing a possible natural reservoir for the cholera bacterium.

Chironomids are the most widely distributed and often the most abundant insect in fresh water[2]. Females deposit egg masses, each containing hundreds of eggs encased in a layer of gelatin, at the water's edge — a convenient location for bacteria to exploit this nutrient-rich substrate.

Two hundred floating *Chironomus* egg masses (Fig. 1a, b) collected from a waste-stabilization pond settled out overnight as thousands of individual eggs (Fig. 1c), most of which did not hatch. To test whether bacteria feeding on and destroying the gelatin matrix might account for this phenomenon, we extracted freshly collected egg masses with a minimal salt-solution medium containing no carbon source[3] in order to isolate and identify any bacteria present.

Four isolates were selected and identified as *Vibrio cholerae* non-O1 non-O139 by standard microbiological tests, as well as by their serotype and fatty-acid profiles. We then collected *Chironomus* egg masses from other waste-stabilization ponds in various regions of Israel and were able to isolate *V. cholerae* from all of these samples.

We inoculated fresh egg masses in a salt-solution medium with 1×10^3 *V. cholerae* per ml. Two controls were run concurrently: egg masses incubated alone under the same conditions, and medium without egg masses inoculated with 1×10^3 per ml *V. cholerae*. We determined growth of *V. cholerae* after 24 h by plating samples on thiosulphate–citrate–bile salt substrate.

The number of bacterial colony-forming units (CFUs) that developed in the first control was always less than 0.1% of that recovered in the treated samples; CFUs recovered in the second control did not change. In the medium containing egg masses as the sole carbon source, *V. cholerae* reached 2×10^6 CFU ml^{-1}. Similar results were obtained when sterilized egg masses were provided as a carbon source. These findings show that egg masses can provide a carbon source to support the development and multiplication of *V. cholerae*.

Although the *V. cholerae* biotypes isolated here are non-pathogenic, it is likely that chironomid egg masses would also be a suitable (and abundant) substrate for the pathogenic *V. cholerae* O1 and O139, assuming that the microhabitat of the pathogenic biotypes is similar[4].

An association has been noted between both viable and 'viable but non-culturable' *V. cholerae* and zooplankton, and copepods have been implicated in the spread of cholera[5,6]. Propagules may be carried by marine zooplankton along the continental seashore, aided by climatic events such as the El Niño Southern Oscillation[7–9]. These results are relevant to the dispersion of pandemics and to the autochthonous existence of *V. cholerae* in endemic locales during periods between epidemics — when there is local build-up of the bacterium but no outbreak of disease. Our findings indicate that chironomid egg masses may serve as an intermediate 'host' reservoir for *V. cholerae*, facilitating its survival and multiplication in freshwater bodies.

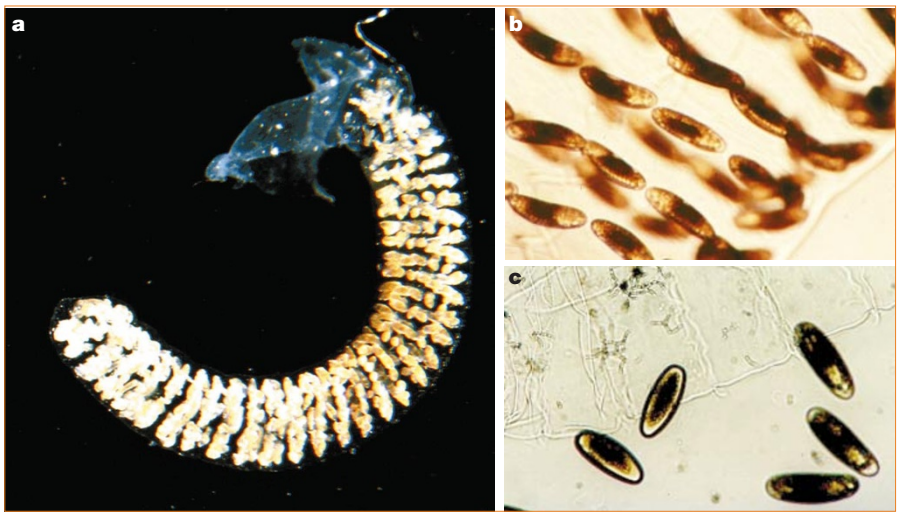

Figure 1 Egg mass of *Chironomus* before and after inoculation with *Vibrio cholerae*. The eggs are arranged in a row, folded into loops to form a spiral, and embedded in a thick, gelatinous cylinder. Egg masses of *Chironomus* spp. are found in freshwater habitats, where they can reach 25 × 5 mm in size and contain about 1,000 eggs. The appearance of several thousand egg masses at one site is not unusual and, in extreme cases, gelatinous layers several centimetres thick are visible from a distance[10,11]. **a,** String-shaped mass of *Chironomus luridus* eggs (original magnification, × 4). **b,** Enlarged portion of the mass, showing the gelatinous matrix. **c,** Enlarged portion 5–10 h after exposure to *V. cholerae*. Eggs are no longer arranged in spiral rows but protrude from the gelatin, which has been partially consumed by the bacteria. Original magnification in **b** and **c**, × 40.

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1. Heidelberg, F. J. *et al. Nature* **406,** 477–484 (2000).
2. Armitage, P., Cranston, P. S. & Pinder, L. C. V. (eds) *The Chironomidae: the Biology and Ecology of Non-Biting Midges* (Chapman & Hall, London, 1995).
3. Echols, H., Garen, A., Garen, S. & Torriani, A. *J. Mol. Biol.* **3,** 425–438 (1961).
4. Lewin, S. M. in *Cholera and the Ecology of* Vibrio cholerae (eds Drasar, B. S. & Forrest, B. D.) 228–254 (Chapman & Hall, London, 1996).
5. Colwell, R. R. & Huq, A. in *Vibrio cholerae and Cholera: Molecular to Global Perspective* (eds Wachsmuth, I. K., Blake, P. A. & Olsvik, Ø.) 117–133 (ASM, Washington DC, 1994).
6. Roszak, D. B. & Colwell, R. R. *Microbiol. Rev.* **51,** 365–379 (1987).
7. Colwell, R. R. *Science* **274,** 2025–2031 (1996).
8. Colwell, R. R. & Huq, A. *J. Appl. Microsc. Symp.* **85** (suppl.), 134–137 (1999).
9. Harvell, C. D. *et al. Science* **285,** 1505–1510 (1999).
10. Nolte, U. *Ent. Scand. Suppl.* **43,** 5–75 (1993).
11. Broza, M., Halpern, M. & Inbar, M. *Wat. Sci.Technol.* **42,** 71–74 (2000).

Carbon fixation

Photosynthesis in a marine diatom

The first stable product of photosynthetic carbon fixation by land plants is either the three-carbon molecule phosphoglycerate (in C_3 plants) or the four-carbon compounds malate or aspartate (in C_4 and CAM (crassulacean-acid metabolism) plants). Reinfelder *et al.* infer that a C_4 biochemical pathway of carbon fixation also operates in marine diatoms[1,2] on the basis of their discovery of the enzyme phosphoenolpyruvate (PEP) carboxylase and of their ^{14}C-tracer results in the marine diatom *Thalassiosira weissflogii*. However, we consider that further analysis is called for to demonstrate that this marine diatom meets all the criteria for C_4 photosynthesis.

C_4 photosynthesis has several features that are essential to CO_2-concentrating mechanisms in general[3], such as an active, photosynthetically driven, CO_2-capture system (PEP carboxylase), an intermediate pool of captured CO_2 (C_4 acids, such as malate), a mechanism to release CO_2 from this pool (decarboxylation of C_4 acids) and a compartment in which to concentrate CO_2 around ribulose 1,5-bisphosphate carboxylase–oxygenase (Rubisco) and to reduce its leakage (for example, the bundle sheath or a functionally equivalent structure). We question whether all these fea-

tures have so far been adequately demonstrated in *T. weissflogii*.

Considering first the nature and location of the carboxylase and decarboxylase, Reinfelder *et al.* suggest[1] that the significance of PEP carboxylase in diatoms may have been missed because of lack of carbon limitation during cell culture. In many studies, however, the dissolved CO_2 concentration was probably the least well controlled growth condition[4], particularly for enzyme assays in which the requirement for algal biomass outweighed other considerations. Earlier reports of PEP carboxylase activity in diatoms were probably due in fact to PEP carboxykinase activity[5,6]. The PEP carboxylase activity found by Reinfelder *et al.* in the chloroplast (53% of total) in the Percoll-purified chloroplast fraction of *T. weissflogii*[1], and the finding of 22% Rubisco activity in the soluble fraction, suggest considerable cross-contamination of the preparations, which would cast doubt on the localization of PEP carboxykinase.

Reinfelder *et al.* also base their conclusions on ^{14}C-tracer kinetics. The shortest labelling time they observed using ^{14}C is 5 s, with much higher labelling of malate than has previously been found in diatoms[5]. However, even the 65% labelling by ^{14}C they report in the C_4 (β-carboxyl) position of malate is not sufficient to justify designation as C_4 biochemistry because secondary carboxylation of labelled PEP, arising from labelled phosphoglycerate generated by Rubisco, can occur.

One crucial diagnostic test for C_4 biochemistry is whether the label on malate extrapolates back to 100% at time zero. However, the two points provided in the data of Reinfelder *et al.*[1] are insufficient to establish a time course. The brown seaweed *Ascophyllum nodosum* (Phaeophyceae) is closely related to diatoms: it has a similar C_4-like photosynthetic physiology[7] and shows significant ^{14}C-labelling of C_4 compounds[8]. But under conditions of inorganic-carbon saturation (sea water), the initial ^{14}C-incorporation product after 1–2 s of photosynthesis is phosphoglycerate[7]. Also, C_4 plants that use PEP carboxykinase as their decarboxylating enzyme use aspartate[9] rather than malate[1] as a substrate.

The finding that phosphoglycerate is the first product of carbon fixation in photosynthesis by *Ascophyllum*[7] and by various diatoms[6] suggests that heterokont algae photosynthesize using a C_3 pathway and that synthesis of C_4 compounds using β-carboxylases (mainly, if not exclusively, PEP carboxykinase) has a ubiquitous anaplerotic role in these organisms. The increased labelling of C_4 compounds relative to phosphoglycerate under conditions of low inorganic carbon is also seen when growth rate is limited by (non-carbon) nutrients or by light[6].

Invoking a role for C_4 photosynthesis to explain the physiological properties and ^{13}C-discrimination values of diatoms seems unnecessary, given the capacity of such cells to accumulate inorganic carbon and hence CO_2 by means of the biophysical CO_2-concentrating mechanism — a process for which there is a large amount of evidence[6,7,10]. Until these issues are unequivocally resolved and the other features of C_4 photosynthesis are demonstrated, we believe that it is premature to designate marine diatoms as C_4 photosynthesizers in the traditional sense.

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1. Reinfelder, J. R., Kraepiel, A. M. L. & Morel, F. M. M. *Nature* **407,** 996–999 (2000).
2. Riebesell, U. *Nature* **407,** 959–960 (2000).
3. Badger, M. R. & Spalding, M. H. in *Photosynthesis: Physiology and Metabolism* (eds Leegood, R. C., Sharkey, T. D. & von Caemmerer, S.) 369 (Kluwer Academic, Dordrecht, 2000).
4. Johnston, A. M. & Raven, J. A. *Marine Ecol. Prog. Ser.* **87,** 517–521 (1992).
5. Beardall, J., Mukerji, D., Glover, H. E. & Morris, I. *J. Phycol.* **12,** 409–417 (1976).
6. Beardall, J. *Aquat. Bot.* **34,** 105–130 (1989).
7. Johnston, A. M. *Can. J. Bot.* **69,** 1123–1132 (1991).
8. Johnston, A. M. & Raven. J. A. *Phycologia* **26,** 159–166 (1987).
9. Sage, R. F. & Monson, R. K. (eds) *C_4 Plant Biology* 596 (Academic, San Diego, 1999).
10. Raven, J. A. *Adv. Bot. Res.* **27,** 85–209 (1997).

Reinfelder et al. *reply* — Johnston *et al.* are not convinced that a C_4 photosynthetic pathway exists in microalgae. The essential feature of C_4 photosynthesis is the fixation of inorganic carbon as a C_4 compound that is subsequently decarboxylated to provide CO_2 as a substrate for Rubisco in the Calvin cycle. It is known that many microalgae incorporate at least some inorganic carbon directly into C_4 compounds such as malate, but the controversy hinges on whether this carbon is released and fixed by Rubisco.

The extrapolation of ^{14}C incorporated into the C_4 position of malate to 100% at time zero, which is proposed as a crucial diagnostic test by Johnston *et al.*, is thus not a direct verification of C_4 photosynthesis. It would be experimentally difficult to determine and is not mathematically well defined — for example, this approach yields an intercept of only 89% in maize, a well-known C_4 plant[1]. A better demonstration is provided by the transfer of ^{14}C from the C_4 compound malate to the C_3 compound phosphoglycerate, the first product of Rubisco, as we have shown. Also, we have recently found (unpublished results) that addition of the C_4 compound oxaloacetate to compensated cultures of *T. weissflogii* triggers immediate production of O_2 in the light, but no O_2 consumption in the dark, which further supports our proposal that this diatom uses C_4 photosynthesis.

Johnston *et al.* also comment on the substrate of PEP carboxykinase and the localization of enzymes. However, oxaloacetate, which is the substrate for PEP carboxykinase, can be produced by oxidation of aspartate or malate. We agree that cellular-fractionation techniques cannot definitively establish how the enzymes of a photosynthetic C_4 pathway are compartmentalized — so far, fractionation results have been confirmed by other techniques in the case of carbonic anhydrase, which has been shown to be located in the cytoplasm of *T. weissflogii* using antibodies.

There is strong evidence that marine diatoms can concentrate inorganic carbon for photosynthesis[2,3], but little to indicate how such a mechanism might work. C_4 pathways are used to accomplish this in multicellular C_4 plants and so may serve the same function in marine diatoms.

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1. Hatch, M. D. & Osmond, C. B. in *Encyclopedia of Plant Physiology* Vol. 3 (eds Stocking, C. R. & Heber, U.) 144–184 (Springer, Berlin, 1976).
2. Raven, J. A. *Adv. Bot. Res.* **27,** 85–209 (1997).
3. Tortell, P. D., Rau, G. H. & Morel, F. M. M. *Limnol. Oceanogr.* **45,** 1485–1500 (2000).

Aerodynamics

Insects can halve wind-turbine power

For no apparent reason, the power of wind turbines operating in high winds may drop, causing production losses of up to 25 per cent[1]. Here we use a new flow-visualization technique to analyse airflow separation over the blades and find that insects caught on the leading edges in earlier low-wind periods are to blame. These potentially catastrophic power glitches can be prevented simply by cleaning the blades.

Unpredictable changes in power levels have been noted on wind farms in California, with power sometimes falling to half the output predicted from the turbine design and generating two or more different power levels at the same wind speed (Fig. 1a). Although this phenomenon (termed 'double' or 'multiple' stalling) has been investigated[2–4], the cause has remained unknown.

One study[5] commissioned by a turbine manufacturer (NEG Micon) used a new invention called a stall flag[6] (patent, Energy Centre of The Netherlands) as a flow-