On sialic acid transport and utilization by *Vibrio cholerae*

The scavenging of host-derived sialic acid is important for a number of human pathogens during colonization of mammalian mucous membranes (Severi *et al.*, 2007). This has been demonstrated for *Vibrio cholerae* (Almagro-Moreno & Boyd, 2009), where the genes for catabolism are encoded on vibrio pathogenicity island 2 (VPI-2) (Jermyn & Boyd, 2002). This genomic element is found only in pathogenic strains of *V. cholerae* and its presence correlates with the ability of vibrio species to utilize sialic acid (Almagro-Moreno & Boyd, 2009). As well as containing the catabolic genes (the *nan* cluster), VPI-2 also contains genes for a tripartite ATP-independent periplasmic (TRAP) transporter, *siaPQM* (VC1777-9), orthologous to the well-characterized sialic acid TRAP transporter from *Haemophilus influenzae* (Severi *et al.*, 2005; Allen *et al.*, 2005; Müller *et al.*, 2006; Johnston *et al.*, 2008; Mulligan *et al.*, 2009). Indeed we have demonstrated that SiaP from *V. cholerae* (VC1779) is a high-affinity sialic-acid-binding protein (Mulligan *et al.*, 2009) and have functionally reconstituted the whole transporter which is active for sialic acid uptake into proteoliposomes (unpublished data).

We were surprised to read the recent publication by Sharma *et al.* (2011) in this journal which claims that a different TRAP transporter is solely responsible for sialic acid transport in this important pathogen. The authors were working on VC1929, a substrate-binding protein (SBP) from an uncharacterized TRAP transporter comprising the VC1927-9 genes. TRAP transporters are commonly found in bacteria and have evolved to recognize a wide range of organic acid substrates (Mulligan *et al.*, 2011), mediated through the interaction of the substrate carboxylate group with a conserved arginine residue in the SBP (Müller *et al.*, 2006; Fischer *et al.*, 2010). Sharma *et al.* (2011) use a bioinformatics approach that leads them

to examine a function of VC1929 as a sialic acid transporter; however, we think this analysis has been presented in a highly selective and misleading way. The PA5167 protein from *Pseudomonas aeruginosa* PAO1 is the most similar protein to VC1929 that has been experimentally characterized, being 56 % identical and 74 % similar at the amino acid level. PA5167 forms part of a characterized C₄-dicarboxylic acid transporter from *P. aeruginosa* (Valentini *et al.*, 2011) and the purified binding protein has identical binding properties to the archetypal C₄-dicarboxylate-binding protein DctP from *Rhodobacter capsulatus* (G. Thomas and D. Kelly, unpublished data). Sharma *et al.* report that VC1929 has '54 % homology' to a sialic acid TRAP SBP from *Aggregatibacter aphrophilus* (although the sequence is not reported it must be NT05HA_0544), yet the identity is actually only 33 % (the similarity is 54 %, but in the alignments to the *Pseudomonas* SBP they report the % identity and not the % similarity to indicate the '% homology'). The SiaP clade of TRAP SBPs, which contains the *A. aphrophilus*, and the *H. influenzae* and *V. cholerae* SiaP proteins (HI0146 and VC1779, respectively), are distinct and distant from the larger C₄-dicarboxylate clade in which VC1929 clearly sits (see the HOGENOME phylogenetic tree for protein family HBG662660). Also, the VC1927-9 genes are adjacent to two genes encoding proteins (VC1925/VC1926) homologous to the DctS/DctR two-component sensor-regulator pair that sense C₄-dicarboxylates, further supporting the putative role in C₄-dicarboxylate transport. Hence, our

bioinformatics analysis of VC1929 suggests that it is likely to be specific for C₄-dicarboxylates, namely succinate, malate and fumarate and not sialic acid.

Sharma *et al.* (2011) then present growth data for strain CD11, which contains a frameshift in VC1929, which shows that this strain is incapable of growth on sialic acid. The data are remarkable as the data presented for the culture of CD11, which has a starting OD₆₀₀ of around 0.08, stays absolutely the same through 19 data points charting 19 h of growth. To our eyes, as experimental bacteriologists, this does not resemble a biological sample in its behaviour, which will always present some small variation in the OD reading over 19 hourly data points when the values are non-zero. However, the authors do not present the details of the growth medium or the growth temperature and there are no error bars on the growth plots which are reported to have been done in triplicate. Given the poor nature of these data and the lack of any other data to support the function of VC1929 in sialic acid transport, we favour our hypothesis that it is the VC1777-9 genes in the VPI-2 island that encode the sole functional sialic acid transporter in this human pathogen. Other data in the paper including the infant mice experiments also have no error bars and have been done only once and should show the exact numbers of mortalities after 96 h.

The linkage by Sharma *et al.* (2011) of VC1929 to a protein that functions as a mannose-sensitive haemagglutinin is based on mapping an abundant soluble protein of around 33 kDa that is missing in the

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proteome of *V. cholerae* in a non-adhering mutant strain CD11 (Jacob *et al.*, 1993); however, no data have been presented by Sharma *et al.* (2011) to demonstrate that VC1929 is the protein missing in this strain. In their recent report, they identify a frameshift in VC1929 which would account for the loss of a functional VC1929 protein in this strain, yet in their original publication (Jacob *et al.*, 1993), the full-length 33 kDa protein band can be seen clearly in CD11 extracts when the protease inhibitor PMSF is added to their sample, suggesting that in this strain, VC1929 is less stable than in the wild-type, rather than it being truncated by a frameshift. Together, these data actually suggest that VC1929 is not the 33 kDa protein identified in their original study and if we apply Occam's razor, it is most likely to be a simple C₄-dicarboxylate binding protein from a TRAP transporter. In conclusion, all our work points to the VPI-2-encoded genes as being those involved in sialic acid transport and utilization and our experience with the functional characterization of diverse TRAP transporters (Thomas *et al.*, 2006; Mulligan *et al.*, 2011) suggests that VC1929 is the SBP component of a C₄-dicarboxylate transporter in *V. cholerae*.

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Authors' response: on sialic acid transport and utilization by *Vibrio cholerae*

At the outset we agree and concur with data generated by Boyd and colleagues that a cluster of genes situated on *vibrio* pathogenicity island 2 (VPI-2) may be involved in transport and catabolism of sialic acid in *Vibrio cholerae*. We have not contradicted but acknowledged their contribution in our recent publication (Sharma *et al.*, 2011). While we were analysing the nature of the defect in the poorly adhesive and non-colonizing mutant CD11 by using SDS-PAGE, we consistently observed the lack of a protein in the mutant compared with the parent strain. The protein was subjected to amino acid sequence determination, which indicated that it was VC1929 (DctP) and the annotated function was transport of C₄-dicarboxylates. We examined the growth of parent and mutant strains with C₄-dicarboxylates and found no difference (Sharma *et al.*, 2011). Further bioinformatics analysis suggested homology to the sialic acid-binding protein SiaP of *Aggregatibacter aphrophilus* and led us to examine growth with sialic acid as the sole carbon source. We are not interested in sialic acid transport and catabolism, but in light of the above Comment, it would be interesting to do the following: (1) construct a DctP knockout and observe growth with glucose, C₄-dicarboxylates and sialic acid; (2) check the binding of DctP to sialic acid, since recombinant DctP can be produced in bulk; (3) screen a *TnphoA* insertion library and analyse periplasmic/secretory mutants for transport and catabolism of sialic acid.

The growth experiment was carried out in M9 minimal medium (Miller, 1972), as mentioned in Methods, and measured by using a Bioscreen growth analyser. The bacterial culture was loaded into this auto-analyser and programmed; the machine delivers the data at the end. There is no possibility of human error during the incubation period. With due respect to bacteriologists, the subtle variation around 0.08 cannot be viewed in Fig. 5 of our manuscript unless the raw data are looked at.