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Effects of ISSo*2* Insertions in Structural and Regulatory Genes of the Trimethylamine Oxide Reductase of *Shewanella oneidensis*

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We have isolated three *Shewanella oneidensis* mutants specifically impaired in trimethylamine oxide (TMAO) respiration. The mutations arose from insertions of an ISSo*2* element into *torA*, *torR*, and *torS*, encoding, respectively, the TMAO reductase TorA, the response regulator TorR, and the sensor TorS. Although TorA is not the sole enzyme reducing TMAO in *S. oneidensis*, growth analysis showed that it is the main respiratory TMAO reductase. Use of a plasmid-borne *torE′-lacZ* fusion confirmed that the TorS-TorR phosphorelay mediates TMAO induction of the *torECAD* operon.

Trimethylamine oxide (TMAO) is a widespread osmoprotective constituent of aquatic animals (17) which can be used by many bacteria as an alternative terminal electron acceptor (3). Reduction of TMAO to the volatile compound trimethylamine generally involves molybdenum-containing enzymes of the same family (6). However, based on their substrate specificity, these enzymes can be divided into two subfamilies: the TMAO reductase and the dimethyl sulfoxide (DMSO) reductase groups. The TMAO reductase enzymes are highly specific, since they can reduce exclusively TMAO as a natural compound or related artificial compounds (13). These enzymes, called TorA, were found in particular in the periplasm of *Escherichia coli* and *Shewanella* species (6, 10, 20). The DMSO reductases, called DorA or DmsA, can reduce a wide range of N- and S-oxide compounds, including DMSO and TMAO (25, 26). They are present in many species, including enterobacteria and *Rhodobacter* species (19).

It has been recently shown in *E. coli* that the TorA protein binds to the pentaheme *c*-type cytochrome TorC (8). The latter is anchored to the inner membrane and allows electron transfer between the membranous quinone pool and the periplasmic terminal enzyme TorA. In *E. coli* and *Shewanella* species, the genes encoding the components of the TMAO reductase system are clustered in the *torCAD* and *torECAD* operons, respectively (6, 10, 20). Three regulatory genes called *torR*, *torS*, and *torT* are found near the *tor* operons. A detailed analysis of the Tor regulatory elements of *E. coli* revealed that TorS is the transmembrane sensor that detects the presence of TMAO in the medium and monitors the proper maturation of TorC (9, 15). TorS contains three phosphorylation sites and transphosphorylates the response regulator TorR by a four-step phosphorelay under inducing conditions (14). Once phosphorylated, TorR activates *torCAD* expression (2). TorT is a periplasmic protein essential for *tor* operon induction, but its precise role remains unclear (16).

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Genes of *Shewanella oneidensis* encoding proteins homologous to TorS, TorR, and TorT of *E. coli* were identified and called *torS*, *torR*, and *torT* (10). However, as no *S. oneidensis* mutant was available, their regulatory functions were investigated by reconstitution experiments in *E. coli*. This approach showed that the three proteins seem to be essential for induction of the *tor* operon of *S. oneidensis*.

Isolation of *S. oneidensis* mutants specifically affected in TMAO respiration. To isolate mutations affecting the main TMAO respiratory system of *S. oneidensis*, we plated MR1-R cells (Table 1), potentially mutagenized by Tn*5* random insertions (4), onto solid rich medium containing 50 mM TMAO and the appropriate antibiotics, and the cells were incubated anaerobically for 36 h at 30°C. Among thousands of clones, we isolated 22 small colonies. To distinguish between pleiotropic and specific mutations, we tested whether these potential mutants were still able to grow normally on rich medium either in aerobiosis or in anaerobiosis in the presence of nitrate (20 mM). Of the 22 clones, only 3 formed large colonies in the presence of oxygen or nitrate. The other 19 clones were not studied further, and we focused our analysis on the three clones that were potentially defective for the main TMAO respiratory system. As shown in Fig. 1, the three mutant strains (SOA-1, SOS-2, and SOR-3) grew more slowly than reference strain MR1-R in anaerobiosis with TMAO, whereas all four strains grew at the same low rate in the absence of TMAO. Interestingly, SOS-2 grew significantly more rapidly than mutants SOA-1 and SOR-3 in the presence of TMAO, while the latter two grew at almost the same low rate with or without TMAO. These results show that SOA-1 and SOR-3 are almost deficient for TMAO respiration, whereas SOS-2 is affected to a lesser extent.

Location and nature of the mutations. As shown previously (10), the putative TMAO reductase of *S. oneidensis* is a large molybdoenzyme called TorA, and the encoding gene is located in the *torECAD* operon of the *tor* locus (Fig. 2A). To check whether TorA was still present in the three mutant strains, we prepared crude extracts (6) from the mutant and the reference strains anaerobically grown overnight in the presence of TMAO. Equivalent amounts of nonheated samples were then loaded on a sodium dodecyl sulfate-polyacrylamide gel, and

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic	Reference or source
S. oneidensis MR1	Wild type; formerly *Shewanella putrefaciens* MR1 ATCC 700550	23
MR1-R	Rifampin-resistant derivative of MR1	G. De Luca
SOA-1	*torA*::ISSo*2* mutant	This work
SOS-2	*torS*::ISSo*2* mutant	This work
SOR-3	*torR*::ISSo*2* mutant	This work
Plasmids		
pACYC184	Cloning vector with a p15A origin of replication	5
pGE593	Operon fusion vector with a pBR origin of replication	7
pBAD33	Vector containing pBAD promoter with a p15A origin of replication	11
$pSTR_{SO}$	*torSTR* sequence from *S. oneidensis* cloned into pBAD33	10
$pBTorA_{SO}$	*torA* sequence from *S. oneidensis* cloned into pBAD33	This work
$pPTor_{SO}7$	*torE* promoter (−84 to +119)[a] from *S. oneidensis* cloned into pGE593	10
pElacZ	*torE'-lacZ* fusion of $pPTor_{SO}7$ cloned into pACYC184	This work

[a] Nucleotide positions relative to the transcription start site of *torE*.

the TMAO reductase active bands were revealed after electrophoresis (Fig. 2B). As expected, an intense band was present for strain MR1-R, and, based on its mobility, it probably corresponds to the TorA enzyme. Strikingly, this active band was missing in SOA-1 and was very faint in SOR-3. Although a band corresponding to TorA was clearly present in SOS-2, its intensity was very low compared to that of strain MR1-R. These results are consistent with the growth properties of the mutant strains, since mutants SOA-1 and SOR-3, which produce at best a very small amount of TorA, grew very slowly in anaerobiosis with TMAO, whereas, under the same conditions, SOS-2, which contains small but significant amounts of TorA, grew more rapidly than SOA-1 or SOR-3. Figure 2B also shows the presence of a second TMAO reductase enzyme migrating just underneath TorA in the four strains. Since this enzyme displayed strong DMSO reductase activity (data not shown), we propose that it is a DMSO reductase able to reduce N- and S-oxide substrates, including TMAO. However, it did not allow efficient TMAO respiration under our experimental conditions.

To check the possibility that the mutations arose from a Tn*5* insertion within *tor* genes, we amplified by PCR with appropriate primers each of the seven genes of the *tor* locus (data not shown). Surprisingly, this simple approach revealed that an insertion of about 1.2 kb was present within the *torA*, *torS*, and *torR* genes for SOA-1, SOS-2, and SOR-3, respectively. Although it proved that *torA*, *torS*, and *torR* were disrupted in these strains, this result was unexpected, since the size of Tn*5* (5.8 kb) is larger than that observed in the mutants. To define the precise positions and the nature of the insertions, we sequenced each of the mutated genes. The three insertions correspond to the same ISSo*2* element (Fig. 2), which belongs to the IS*3* family (18), and this element was found in four intact copies and one partly deleted copy on the chromosome of strain MR1 (12). The insertions of this mobile element within the *tor* genes led to a duplication of four nucleotides of the target DNA. As indicated in Fig. 2C, *torA*, *torS*, and *torR* were disrupted at positions +1714, +716, and −18 from the start codon in SOA-1, SOS-2, and SOR-3, respectively. The fact that the mutations in the *tor* genes occurred by insertions of the ISSo*2* endogenous element rather than by Tn*5* transposition is puzzling, since Tn*5* mutagenesis has been used successfully in *S. oneidensis* (4).

To confirm that the phenotypes of the mutants came from the *tor* gene disruptions, we amplified the *torA* gene and the *torSTR* gene cluster by PCR and cloned them into pBAD33, a replicative plasmid for *S. oneidensis* (22). Plasmids $pBTorA_{SO}$ and $pSTR_{SO}$ were then introduced by electrotransformation into strains SOA-1 and SOS-2 or SOR-3, respectively. Electrotransformation was performed as previously described (22), except that the solution containing 1 M sorbitol was buffered with Tris-HCl, pH 7.6. As shown in Fig. 1, presence of the

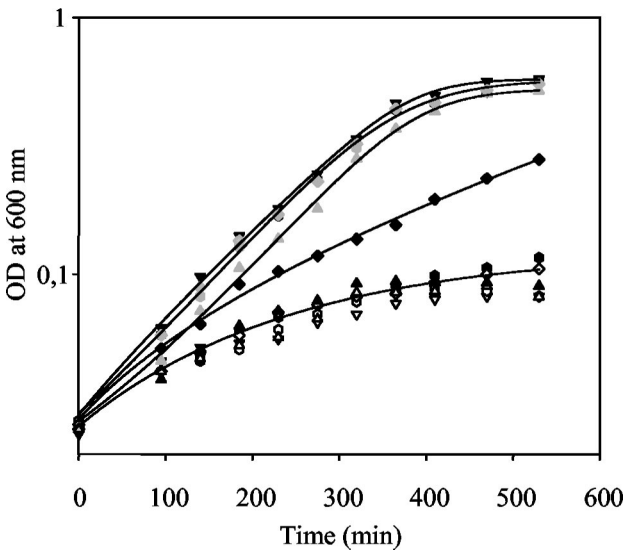

FIG. 1. Anaerobic growth profiles of *S. oneidensis* MR1-R and *tor* mutants carrying different plasmids. Strains MR1-R (▽ and ▼), SOA-1 (△ and ▲), SOS-2 (◇ and ◆), and SOR-3 (□ and ■), all carrying pBAD33, were grown at 30°C in Luria-Bertani rich medium complemented with 40 mM L-lactate and 20 mM HEPES (24), in either the absence (white symbols) or presence (black symbols) of 50 mM TMAO. The grey symbols correspond to SOS-2 (diamonds) and SOR-3 (squares), both carrying $pSTR_{SO}$, and SOA-1, carrying $pBTorA_{SO}$ (triangles), grown in the presence of TMAO. For the latter, arabinose (0.001%) was added. Growth was monitored at 600 nm. Data are typical of at least three independent experiments.

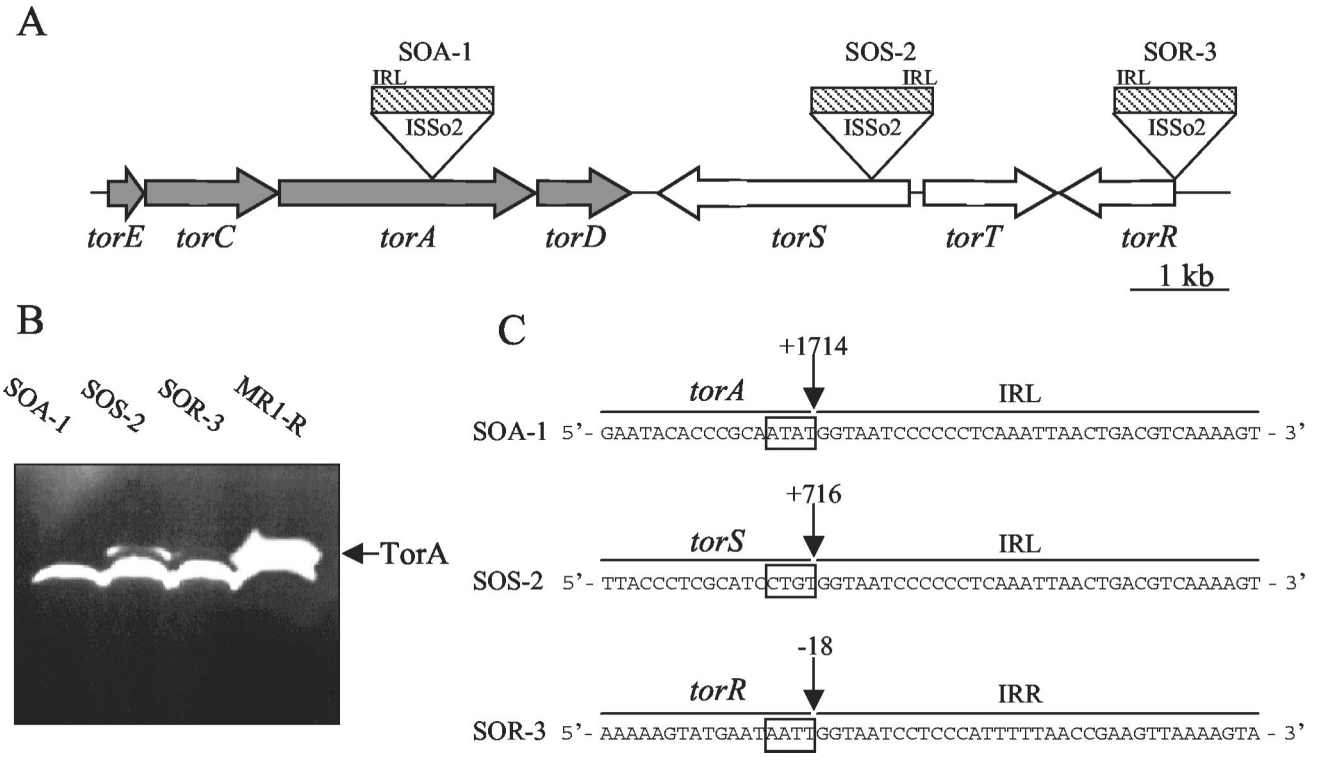

FIG. 2. (A) Schematic representation of the positions of the ISSo*2* insertion in *torA*, *torS*, and *torR*. The large arrows show the location and orientation of the *tor* genes. The hatched boxes symbolize the insertion elements; the position of the left inverted repeat (IRL) is indicated. (B) In-gel TMAO reductase activity. Nonheated crude extracts (75 μg) of SOA-1, SOS-2, SOR-3, and MR1-R were loaded on a sodium dodecyl sulfate–7.5% polyacrylamide gel. After electrophoresis, the gel was stained for methyl viologen-TMAO reductase activity (6). (C) Insertion sites of the ISSo*2* element in *torA*, *torS*, and *torR*. The vertical arrows indicate the insertion sites, and boxes indicate the four nucleotides which were duplicated during ISSo*2* insertion. Left and right inverted repeats (IRL and IRR) show the orientation of the mobile element. Numbering is relative to the translation start point of each gene.

hybrid plasmids complemented the defect of the mutant strains in anaerobic growth with TMAO, confirming that the disrupted genes are involved in the main TMAO respiratory pathway of *S. oneidensis*.

Regulation of *torECAD* operon expression. Based on reconstitution experiments in *E. coli* (10) and on the results described above, we suspected that the *torR* and *torS* gene products play a key role in the induction of the *tor* structural operon. To study expression of the *tor* operon directly in *S. oneidensis*, we amplified and cloned the entire *torE′-lacZ* DNA fragment of pPTor$_{SO}$7 (from position −29 to +3335 relative to the *Eco*RI cloning site) into pACYC184. The resulting plasmid (pElacZ) was introduced into the mutant and reference strains, and β-galactosidase activities were measured on whole cells by the method of Miller (21). As shown in Table 2, in anaerobiosis, the β-galactosidase activities of the plasmid-borne *torE′-lacZ* fusion increased almost 40-fold when TMAO was added for the reference and the SOA-1 strains. In contrast, the activities of the fusion were very low and did not significantly increase upon TMAO addition for SOS-2 and SOR-3. These results confirm that TMAO strongly induces *tor* operon expression and that this induction requires the *torS* and *torR* gene products. No *torA* autoregulation was observed, since the activity levels were similar in the reference and the *torA* strains under inducing conditions. Interestingly, although expression of the plasmid-borne *torE′-lacZ* fusion was very low in SOS-2, it was even lower in SOR-3. This last point supports the idea that expression of the *tor* operon is not entirely locked in SOS-2 and agrees with the fact that a small amount of TorA enzyme was still present in the *torS* strain (Fig. 2). An explanation is that TorS, like many sensors, can dephosphorylate its partner in the absence of any inducer (27) and can thus remove phosphate of any origin from TorR. As in *E. coli* (1), dephosphorylation of TorR could involve a reverse phosphotransfer from TorR to TorS.

In conclusion, isolation and characterization of various mutations in the *tor* genes of *S. oneidensis* allowed us to confirm that the TorS-TorR phosphorelay is involved in the control of

TABLE 2. Expression of the plasmid-borne *torE′-lacZ* fusion in *S. oneidensis* strains

Strain carrying pElacZ[a]	β-Galactosidase activity[b]	
	+ TMAO	− TMAO
MR1-R	6,240 ± 430	157 ± 23
SOA-1	6,030 ± 290	153 ± 14
SOS-2	350 ± 28	340 ± 17
SOR-3	215 ± 30	166 ± 22

[a] All strains were grown anaerobically in the presence (+) or absence (−) of TMAO.

[b] β-Galactosidase activities are expressed in Miller units. Values are averages of at least three independent experiments. Standard deviations were <15%.

torECAD in response to TMAO availability and to establish that TorA is the major TMAO reductase respiratory enzyme of *S. oneidensis*.

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