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The role of the outer membrane in formaldehyde tolerance in *Escherichia coli* VU3695 and *Halomonas* sp. MAC

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To investigate the mechanism of formaldehyde tolerance in Gram-negative bacteria, two formaldehyde-tolerant strains, *Escherichia coli* VU3695 and *Halomonas* sp. MAC (DSM 7328), and formaldehyde-sensitive revertants obtained by ethidium bromide or novobiocin treatment were studied. The presence of high levels of formaldehyde dehydrogenase activity alone proved insufficient to confer tolerance to high formaldehyde concentrations, as shown by the high activity displayed by formaldehyde-sensitive revertants of *Halomonas* MAC. Moreover, formaldehyde-tolerant strains also proved to be tolerant to high concentrations of acetaldehyde and glutaraldehyde, which are not oxidized by formaldehyde dehydrogenase. Treatment with sublethal concentrations of EDTA rendered the resistant strains highly sensitive to formaldehyde without affecting the activity of formaldehyde dehydrogenase. Comparison of the outer membrane proteins of formaldehyde-resistant strains with those of their sensitive revertants showed the presence of at least one additional high molecular mass protein in the tolerant strains. It is concluded that formaldehyde tolerance in the bacteria studied depends on the composition and structure of the outer membrane.

Keywords: *Escherichia coli*, *Halomonas* sp., formaldehyde, outer membrane, tolerance

INTRODUCTION

In recent years a number of reports have appeared on the occurrence of bacteria resistant to high concentrations of formaldehyde. Strains of *Escherichia coli* and *Serratia marcescens* tolerating formaldehyde concentrations of 500 and 200 p.p.m., respectively, have been isolated (Kaulfers & Marquardt, 1991; Kaulfers & Laufs, 1985). Likewise, highly formaldehyde-tolerant strains of *Pseudomonas putida* and *Halomonas* sp. have been characterized (Azachi *et al.*, 1995; Kato *et al.*, 1984, 1986).

One of the factors that may play a role in conferring formaldehyde tolerance in bacteria is the presence of intracellular enzymes metabolizing formaldehyde. Thus, high levels of activity of an NAD- and glutathione-dependent formaldehyde dehydrogenase (EC 1.2.1.1) were demonstrated in formaldehyde-resistant strains of *E. coli*, *S. marcescens*, and *Halomonas* sp. (Azachi *et al.*, 1995; Kaulfers & Marquardt, 1991; Kaulfers & Laufs, 1985). Additional enzymes, such as formaldehyde dismutase (EC 1.2.99.4) and dye-linked formaldehyde dehydrogenase (EC 1.2.99.3) may be involved in the detoxification of formaldehyde in other micro-organisms (Kato *et al.*, 1984, 1986, 1988). In certain bacteria at least, the property of formaldehyde tolerance may be plasmid-mediated (Kaulfers & Brandt, 1987; Kaulfers & Laufs, 1985).

In the present work we investigated the possible role of the outer membrane of Gram-negative bacteria in formaldehyde resistance. The outer membrane is the primary barrier that formaldehyde molecules encounter when interacting with these bacteria. The outer membrane has been shown to act as a molecular sieve which determines, mainly on the basis of molecular size, whether or not a substrate can enter the cell (Lieve, 1974; Nikaido & Vaara, 1985). To our knowledge, the role of the outer membrane in formaldehyde tolerance has not previously been investigated. We present evidence that the properties of the outer membrane are of prime importance in conferring formaldehyde tolerance in *E. coli* and in a *Halomonas* isolate.

METHODS

Bacterial strains and culture conditions. The formaldehyde-tolerant strain *Escherichia coli* VU3695 (Kaulfers & Marquardt, 1991), which harbours a 94 kb plasmid, was grown at 37 °C with constant gyratory shaking at 150 r.p.m. in Luria broth medium (LB) (Sambrook *et al.*, 1989) or in nutrient broth (NB). Formaldehyde-sensitive revertants (strain VU3695-21 and VU3695-31) were isolated by random mutagenesis in the presence of ethidium bromide, as described by Kaulfers & Brandt (1987). *Halomonas elongata* ATCC 33173 and *Halomonas* sp. MAC (DSM 7328), which show high levels of formaldehyde resistance (Azachi *et al.*, 1995), were grown at 30 °C with constant gyratory shaking at 150 r.p.m. in LB or NB medium supplemented with 10% (w/v) NaCl. Three revertant strains of *Halomonas* MAC, which itself harbours a 15 kb plasmid, were obtained by treatment with novobiocin as described below: MAC-29, a formaldehyde-tolerant strain lacking the plasmid, MAC-6, a formaldehyde-sensitive strain lacking the plasmid, and MAC-2, a sensitive strain containing the plasmid.

Growth inhibition by formaldehyde, acetaldehyde and glutaraldehyde. Overnight cultures of *E. coli* VU3695, and *Halomonas* MAC and mutants as indicated above were grown in NB medium and 10% NaCl-supplemented NB medium, respectively. They were then harvested by centrifugation (12000 ***g*** at 4 °C for 10 min). Cells were resuspended in the growth medium to an OD_{540} of 0·03 and cultures were amended with formaldehyde [37%, v/v, solution containing 10%, v/v, methanol (Merck)], or acetaldehyde or glutaraldehyde at concentrations up to 500 p.p.m. Triplicate portions (5 ml in 20 ml test tubes) were incubated aerobically at 37 or 30 °C, respectively, and after 48 h the OD_{540} was measured.

Effect of formaldehyde and EDTA on cell viability. Cells were harvested in the early to mid-exponential growth phase by centrifugation (12000 ***g*** for 10 min at 4 °C). To test the effect of sublethal EDTA concentrations on sensitivity to killing by formaldehyde, cells were washed twice with 10 mM Tris/HCl buffer, pH 8·0, containing 0·9% NaCl (for *E. coli*) or 10% NaCl (for *Halomonas*), and resuspended to a final OD_{540} of 0·055 in 30 ml of the appropriate buffer containing 1 mM or 0·5 mM EDTA, respectively. In control experiments EDTA was omitted. Suspensions were incubated at the growth temperature for 1 h, followed by washing twice in buffer without EDTA and suspension in buffer. Formaldehyde (25 or 1000 p.p.m.) was added, and after 1 h of incubation at the growth temperature the cultures were decimally diluted and the number of c.f.u. counted on LB agar with or without 10% NaCl as required, and compared with the number in the absence of formaldehyde. Similar experiments were performed with acetaldehyde (15 or 500 p.p.m.) or glutaraldehyde (25 or 1000 p.p.m.).

Isolation and characterization of outer membrane and cytoplasmic proteins. The outer membrane and the cytoplasmic fraction were isolated according to the following protocol, based in part on methods described by Hancock & Nikaido (1978), McGavin *et al.* (1990) and Wang & Thomson (1990). Cultures (500 ml) in the late exponential growth phase were supplemented with NaCl and Tris/HCl buffer, pH 7·3, to a final concentration of 33 mM each. After 10 min incubation at 25 °C, cells were harvested by centrifugation and resuspended in 33 mM Tris/HCl buffer, pH 7·3, to a density of 100 mg wet cell wt ml^{-1}. An equal volume of buffer consisting of 33 mM Tris/HCl, 40% (w/v) sucrose and 2 mM EDTA, pH 7·3, was added, and after an additional centrifugation, cells were suspended in ice-cold 1 mM $MgCl_2$ (50 mg wet wt ml^{-1}) and incubated at −20 °C for 10 min, followed by 10 min centrifugation at 10000 ***g*** at 4 °C. Cells were washed once in PBS (3·3 mM NaH_2PO_4, 6·7 mM Na_2HPO_4, 126·5 mM NaCl, pH 7·0), resuspended in the same buffer, and disrupted by two passages through a French press cell in the cold. The lysate was centrifuged at 16300 ***g*** for 30 min at 4 °C. Ten millilitres of the supernatant was subsequently centrifuged at 30500 ***g*** for 2·5 h at 4 °C and the supernatant was collected (cytoplasmic fraction). Another 10 ml portion of the supernatant was diluted by the addition of 10 ml of Tris buffer and 6 ml was layered on top of a sucrose step gradient consisting of 1 ml 70% sucrose in Tris buffer on the bottom and 6 ml of 15% sucrose in Tris buffer on top, and the tubes were centrifuged at 121500 ***g*** for 2·5 h at 4 °C. The bottom 2 ml of each gradient was removed, and each 2 ml was applied to another sucrose density gradient containing 5 ml of 60% sucrose in Tris buffer in the bottom layer and 5 ml of 47% sucrose in Tris buffer in the top layer. Tubes were centrifuged for 6 h at 121500 ***g***. The lower outer membrane band was collected, diluted in distilled water and centrifuged at 30500 ***g*** for 2·5 h at 4 °C. The resulting pellet was washed twice with PBS and resuspended at 4 °C in 10 ml 50 mM Tris/HCl containing 1 mM $MgCl_2$ and 0·2 mM DTT, pH 8·0.

Protein was determined by the method of Bradford (1976), with BSA as a standard.

Proteins were separated by electrophoresis in SDS-PAGE gels [10% (w/v) acrylamide], and stained with Coomassie brilliant blue G250, using standard methods (Hancock & Carey, 1979). The molecular masses of the proteins were estimated by using low-range prestained standards (Bio-Rad Laboratories), which included the following proteins: phosphorylase B (142·9 kDa), bovine serum albumin (97·2 kDa), ovalbumin (50 kDa), carbonic anhydrase (35 kDa), soybean trypsin inhibitor (29·7 kDa) and lysozyme (21·9 kDa).

Plasmid isolation, transformation and curing. Plasmid DNA was isolated as described by Birnboim & Doly (1979) or using the QIAGEN plasmid kit (Hildan). Plasmids or their *Nhe*I digests were analysed on 0·8% agarose gels, followed by ethidium bromide staining and UV-visualization (Sambrook *et al.*, 1989). The molecular masses of the plasmid fragments were estimated by comparison with DNA ladder fragments. Plasmid transformation was performed by chemical treatment (Sambrook *et al.*, 1989) or by electroporation (Diver *et al.*, 1990). *Halomonas* MAC was cured of its plasmid by growing the bacteria for 24 h, transferring every 8 h in LB medium containing 5 μg novobiocin ml^{-1}. Inoculum size was 1, 5 and 10% (v/v) for the first, second and third transfers, respectively. Bacteria surviving the curing treatment were centrifuged for 10 min at 10000 ***g*** at 4 °C, washed twice with 10% NaCl medium and then plated on 10% NaCl-containing LB agar plates without formaldehyde or with 20 or 50 p.p.m. formaldehyde. Plasmid curing of *E. coli* VU3695 was performed using 1·8 mg ethidium bromide ml^{-1} (Kaulfers & Brandt, 1987).

Formaldehyde dehydrogenase assays. NAD- and glutathione-dependent formaldehyde dehydrogenase was assayed spectrophotometrically as described previously (Azachi *et al.*, 1995; Kaulfers & Marquardt, 1991). The formaldehyde concentration in the reaction mixture was 0·1 mg ml^{-1}.

RESULTS AND DISCUSSION

Effect of formaldehyde, acetaldehyde and glutaraldehyde on cell viability in the presence and absence of EDTA

E. coli strain VU3695 and *Halomonas* MAC tolerated high formaldehyde concentrations and treatment with 1000 p.p.m. formaldehyde for 1 h did not cause a marked decrease in the number of c.f.u. However, when treatment

Table 1. Influence of sublethal EDTA concentrations on the resistance of *E. coli* VU3695 and *Halomonas* MAC to formaldehyde, acetaldehyde and glutaraldehyde

Suspensions of bacteria (about 10^8 cells ml^{-1}) were exposed to different concentrations of aldehydes as indicated, preceded or not preceded by 1 h incubation with 1·0 or 0·5 mM EDTA, respectively. The number of colony-forming cells (c.f.u.) remaining was determined in triplicate after 1 h. Results are means ± SD.

Treatment	Viable counts ($\log_{10}$ c.f.u. ml^{-1})	
	E. coli VU3695	*Halomonas* MAC
None	7·9 ± 0·3	8·1 ± 0·4
EDTA	7·9 ± 0·2	8·1 ± 0·3
Formaldehyde (25 p.p.m.)	8·0 ± 0·4	8·1 ± 0·4
Formaldehyde (1000 p.p.m.)	7·6 ± 0·3	7·6 ± 0·2
EDTA + formaldehyde (25 p.p.m.)	6·0 ± 0·1	6·9 ± 0·2
EDTA + formaldehyde (1000 p.p.m.)	0·5 ± 0·5	5·4 ± 0·1
Acetaldehyde (15 p.p.m.)	7·9 ± 0·4	8·0 ± 0·4
Acetaldehyde (500 p.p.m.)	7·6 ± 0·1	8·1 ± 0·2
EDTA + acetaldehyde (15 p.p.m.)	6·5 ± 0·4	7·5 ± 0·1
EDTA + acetaldehyde (500 p.p.m.)	5·0 ± 0·4	5·0 ± 0·3
Glutaraldehyde (25 p.p.m.)	7·4 ± 0·3	7·6 ± 0·3
Glutaraldehyde (1000 p.p.m.)	6·3 ± 0·3	6·0 ± 0·4
EDTA + glutaraldehyde (25 p.p.m.)	5·8 ± 0·2	6·6 ± 0·2
EDTA + glutaraldehyde (1000 p.p.m.)	0·5 ± 0·5	1·4 ± 1·0

Table 2. MICs of formaldehyde, acetaldehyde and glutaraldehyde in *E. coli* VU3695, *Halomonas* MAC and mutant strains

Strain	MIC (p.p.m.)		
	Formaldehyde	Acetaldehyde	Glutaraldehyde
Halomonas MAC	55	15	100
Halomonas MAC-29	55	15	100
Halomonas MAC-6	20	10	50
Halomonas MAC-2	25	10	50
E. coli VU3695	150	30	150
E. coli VU3695-31	30	10	55
E. coli VU3695-21	35	15	60

with formaldehyde (25 or 1000 p.p.m.) was preceded by incubation with sublethal concentrations of EDTA, a reduction in viable counts of 1·9 and 7·4 $\log_{10}$, respectively, was observed with *E. coli*, and 1·2 and 2·7 $\log_{10}$ with *Halomonas* (Table 1). Addition of EDTA alone did not affect viable counts at the concentrations used. Similar results were obtained when 10% sucrose was included during the incubation to stabilize the cells. The results of these experiments suggest a possible involvement of the outer membrane in formaldehyde tolerance. EDTA treatment removes divalent cations which are essential for the structural integrity of the outer membrane, and causes the release of part of the lipopolysaccharide layer, thereby increasing the permeability of the outer membrane. Thus, EDTA treatment enhanced the susceptibility of *E. coli* and other Gram-negative bacteria to several antibiotics (Spicer & Spooner, 1974; Weiser *et al.*, 1968). EDTA treatment also increased the sensitivity of *E. coli* VU3695 and *Halomonas* MAC to acetaldehyde and glutaraldehyde (Table 1).

Formaldehyde tolerance in *E. coli* VU3695 and *Halomonas* MAC is not due to formaldehyde dehydrogenase alone

The presence of high levels of formaldehyde dehydrogenase activity has been implicated in the mechanism of formaldehyde resistance in *E. coli* and other bacteria

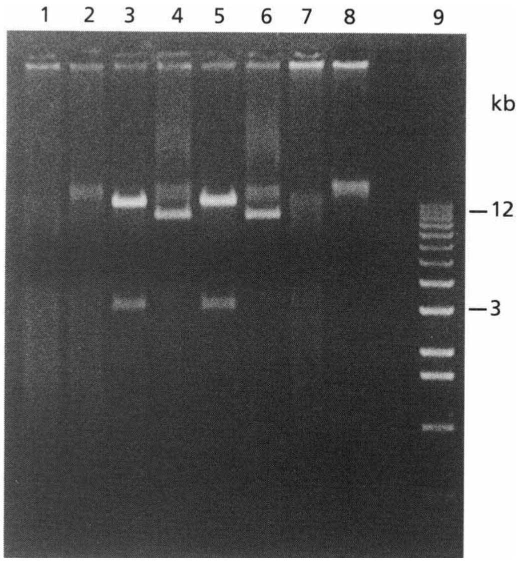

Fig. 1. Agarose gel electrophoresis of intact or *Nhe*I-digested plasmids isolated from *Halomonas* MAC and its revertants. Lanes: 1 and 2, MAC-6; 3 and 4, MAC-2; 5 and 6, wild-type; 7 and 8, MAC-29; 1, 3, 5 and 7, *Nhe*I-digested; 2, 4, 6 and 8, undigested; 9, DNA ladder.

(Azachi *et al.*, 1995; Kaulfers & Marquardt, 1991; Kaulfers & Laufs, 1985). We therefore tested whether treatment with sublethal EDTA concentrations affected formaldehyde dehydrogenase activity. No significant differences were observed in formaldehyde dehydrogenase activity in *E. coli* VU3695 harvested before and after treatment with 1 mM EDTA [150·3 and 150 nmol (mg protein)$^{-1}$ min^{-1} at 25 °C] and in *Halomonas* with and without 0·5 mM EDTA [94·5 and 94 nmol (mg protein)$^{-1}$ min^{-1}]. These results demonstrate that tolerance to high concentrations of formaldehyde cannot be attributed to the presence of high levels of formaldehyde dehydrogenase activity alone.

Additional evidence for the lack of linkage between tolerance to formaldehyde and formaldehyde dehydrogenase activity came from a comparison of the sensitivity of the above strains and revertants to acetaldehyde and glutaraldehyde, two aldehydes that are not oxidized by formaldehyde dehydrogenase. In all cases tolerance toward the three aldehydes was correlated (Table 2), again showing that differences in enzyme activity alone are insufficient to explain differences in aldehyde tolerance.

Formaldehyde tolerance in *Halomonas* MAC is not plasmid-determined

Previous studies with *E. coli* strain VU3695 suggested that its tolerance to formaldehyde is plasmid-mediated, and plasmid-cured cells were found to be formaldehyde-sensitive (Kaulfers & Brandt, 1987). Plasmid analysis in *Halomonas* MAC showed a single band with an apparent size of 15 kb (Fig. 1). We attempted to transform the 15 kb plasmid into the formaldehyde-sensitive *E. coli* strains DH5α and HB101 (Sambrook *et al.*, 1989) and into *Halomonas elongata* ATCC 33173, which is also formaldehyde-sensitive. pBluescript (Stratagene) was used as a positive control. Transformants were plated onto media (LB agar for *E. coli*, LB agar supplemented with 10% NaCl for *Halomonas*) containing 50, 75 or 100 p.p.m. formaldehyde. No formaldehyde-resistant transformants were found. This may have been due to one or more of the following reasons: (i) the plasmid does not possess the correct origin of replication; (ii) formaldehyde resistance is not plasmid-mediated; (iii) the gene(s) present on the plasmid are not expressed as proteins.

In order to discriminate between the above possibilities, we attempted to cure *Halomonas* MAC of its plasmid using a variety of methods (heat and different chemical treatments). Three variant strains were obtained by treatment with novobiocin: MAC-29, a formaldehyde-tolerant strain lacking the plasmid, MAC-6, a formaldehyde-sensitive strain lacking the plasmid, and MAC-2, a sensitive strain containing the plasmid (Fig. 1). All three revertants possessed formaldehyde dehydrogenase activities similar to that of the wild type [93 ± 1 nmol (mg protein)$^{-1}$ min^{-1}]. Thus, the presence of the plasmid was not necessary for formaldehyde tolerance in *Halomonas* MAC, and formaldehyde dehydrogenase activity is probably not mediated by the 15 kb plasmid.

Using ethidium bromide mutagenesis of *E. coli* VU3695 we isolated two formaldehyde-sensitive revertants (strains 31 and 21). These revertants contained the plasmid of the parent strain, but lacked formaldehyde dehydrogenase activity. Interestingly, using the same procedure as Kaulfers & Brandt (1987), we have observed loss of formaldehyde dehydrogenase activity in the presence of the plasmid. It should be noted that ethidium bromide is a mutagenic agent that may damage gene activity both in the chromosome and in the plasmid. Therefore, it cannot be ascertained whether the gene(s) that confer formaldehyde resistance in *E. coli* VU3695 are plasmid-linked or chromosomal.

Formaldehyde tolerance in *E. coli* VU3695 and *Halomonas* MAC involves the outer membrane

The outer membrane is the first barrier encountered by formaldehyde to penetration of Gram-negative bacteria. We therefore investigated whether differences in formaldehyde tolerance of the strains and their sensitive revertants was correlated with differences in the structure of their outer membranes. The outer membrane preparations obtained were found to be free of formaldehyde dehydrogenase activity, showing that contamination with cytoplasmic proteins was insignificant. As in other Gram-negative bacteria (Lidstrom, 1992), the formaldehyde-oxidizing activity in *Halomonas* MAC and *E. coli* VU3695 was found to be located in the cytoplasmic fraction. Comparison of the outer membrane protein profile of *E. coli* VU3695 and its formaldehyde-sensitive revertants 31 and 21 showed that an additional high-molecular-mass protein (apparent molecular mass > 143 kDa) was present in the wild-type strain, but missing in both

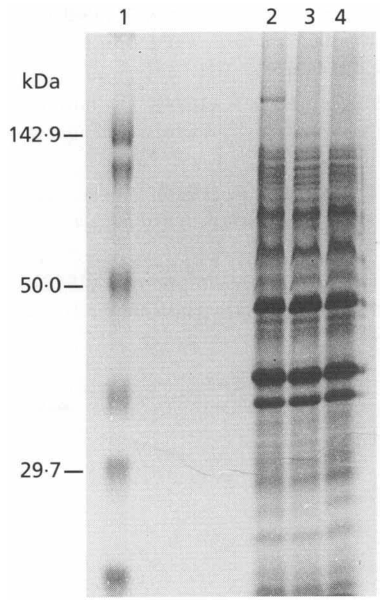

Fig. 2. SDS-PAGE of outer membrane proteins of *E. coli* VU3695 (lane 2) and revertants 31 (lane 3) and 21 (lane 4). Lane 1 contains molecular mass standards.

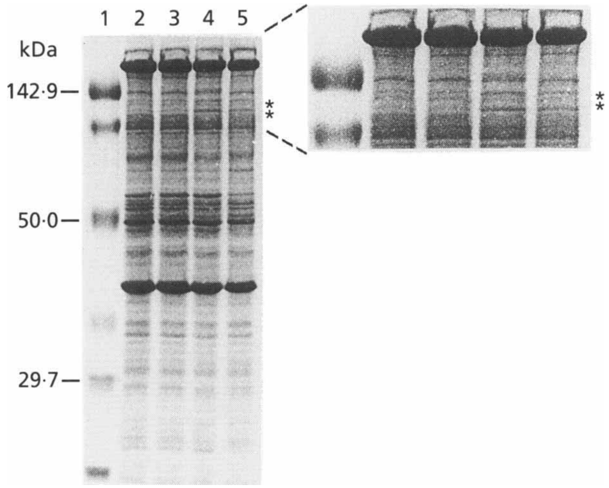

Fig. 3. SDS-PAGE of outer membrane proteins of *Halomonas* MAC (lane 5) and revertants 2 (lane 2), 6 (lane 3) and 29 (lane 4). Lane 1 contains molecular mass standards.

sensitive revertants (Fig. 2). In *Halomonas* MAC, two proteins (apparent molecular mass 98–143 kD) were prominent in both the wild-type and in strain MAC-29 (formaldehyde-tolerant, lacking the plasmid), but were present in reduced amounts in the formaldehyde-sensitive revertants MAC-6 and MAC-2 (Fig. 3).

The above data indicate the importance of the outer membrane as the barrier that protects the cell from formaldehyde, and show that unique proteins may participate in conferring formaldehyde resistance. To some extent the above findings are surprising, as a molecule as small as formaldehyde would be expected to penetrate the outer membrane through porins. We are currently investigating the properties of the proteins involved and the corresponding genes in order to elucidate their function in formaldehyde resistance. We expect that further characterization of the above-described mutants may lead to the identification of the gene(s) involved in formaldehyde tolerance, and an in-depth understanding of the phenomenon. We recently initiated a study to identify and characterize the relevant genes by using random mutagenesis of the resistant mutant MAC-29, lacking the plasmid, with transposon Tn*5*. A number of formaldehyde-sentitive mutants were obtained, which did show formaldehyde dehydrogenase activity, but lacked the ~ 98 kDa protein in their outer membrane (results not shown). One of these mutants was shown to contain a single copy of Tn*5*. Further characterization of this mutant is in progress.

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REFERENCES

Azachi, M., Henis, Y., Oren, A., Gurevich, P. & Sarig, S. (1995). Transformation of formaldehyde by a *Halomonas* sp. *Can J Microbiol* **41**, 548–553.

Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7**, 1513–1523.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein–dye binding. *Anal Biochem* **72**, 248–254.

Diver, M. D., Larry, E. B. & Pamela, A. S. (1990). Transformation of *Pseudomonas aeruginosa* by electroporation. *Anal Biochem* **189**, 75–79.

Hancock, R. E. W. & Carey, A. M. (1979). Outer membrane of *Pseudomonas aeruginosa*: heat and 2-mercaptoethanol-modifiable proteins. *J Bacteriol* **140**, 902–910.

Hancock, R. E. W. & Nikaido, H. (1978). Outer membranes of gram-negative bacteria. XIX. Isolation from *Pseudomonas aeruginosa* PAO1 and use in reconstitution and definition of the permeability barrier. *J Bacteriol* **136**, 381–390.

Kato, N., Kobayashi, H., Shimao, M., & Sakazawa, C. (1984). Properties of formaldehyde dismutation catalyzing enzyme of *Pseudomonas putida* F61. *Agric Biol Chem* **48**, 2017–2023.

Kato, N., Yamagami, N., Shimao, M. & Sakazawa, C. (1986). Formaldehyde dismutase, a novel NAD-binding oxidoreductase from *Pseudomonas putida* F61. *Eur J Biochem* **156**, 59–64.

Kato, N., Miyamoto, N., Shimao, M. & Sakazawa, C. (1988). 3-Hexulose phosphate synthase from a new facultative methylotroph, *Mycobacterium gastri* MB19. *Agric Biol Chem* **52**, 2659–2661.

Kaulfers, P. M. & Brandt, D. (1987). Isolation of a conjugative plasmid in *Escherichia coli* determining formaldehyde resistance. *FEMS Microbiol Lett* **43**, 161–163.

Kaulfers, P. M. & Laufs, R. (1985). Übertragbare Formaldehydresistenz bei *Serratia marcescens*. *Zentralbl Bakteriol Mikrobiol Hyg I Abt Orig B* **181**, 309–319.

Kaulfers, P. M. & Marquardt, A. (1991). Demonstration of formaldehyde dehydrogenase activity in formaldehyde-resistant *Enterobacteriaceae*. *FEMS Microbiol Lett* **79**, 335–338.

Lidstrom, M. E. (1992). The aerobic methylotrophic bacteria. In *The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, pp. 431–445. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer-Verlag.

Lieve, L. (1974). The barrier function of the gram-negative cell envelope. *Ann NY Acad Sci* **235**, 109–127.

McGavin, M., Lam, J. & Forsberg, C. W. (1990). Regulation and distribution of *Fibrobacter succinogenes* subsp. *succinogenes* S85 endoglucanases. *Appl Environ Microbiol* **56**, 1235–1244.

Nikaido, H. & Vaara, M. (1985). Molecular basis of bacterial outer membrane permeability. *Microbiol Rev* **49**, 1–32.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Spicer, A. B. & Spooner, D. F. (1974). The inhibition of growth of *Escherichia coli* spheroplasts by antibacterial agents. *J Gen Microbiol* **80**, 37–50.

Wang, W. & Thomson, J. A. (1990). Nucleotide sequence of the *celA* gene encoding a cellodextrinase of *Ruminococcus flavefaciens*. *Mol Gen Genet* **222**, 265–269.

Weiser, R., Asscher, A. W. & Wimpenny, J. (1968). In vitro reversal of antibiotic resistance by ethylendiamine tetraacetic acid. *Nature* **219**, 1365–1366.

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