Mercuric Reductase Enzymes from *Streptomyces* Species and Group B *Streptococcus*

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Mercury volatilization (Hg^{2+} reductase) activity has been found with Hg^{2+} -resistant isolates of three *Streptomyces* species and with three Hg^{2+} -resistant strains of group B *Streptococcus* from clinical sources in Japan. Hg^{2+} reductase activities in crude cell extracts showed the temperature sensitivity, the requirement for an added thiol compound and the characteristic dependence on NAD(P)H cofactors of similar enzymes isolated from other bacteria.

INTRODUCTION

Hg^{2+} -resistant bacteria have been reported in a wide variety of Gram-negative and Gram-positive species including enterics, pseudomonads, *Staphylococcus aureus*, *Bacillus*, *Yersinia*, *Mycobacterium* and *Thiobacillus* (for reviews see Summers & Silver, 1978; Robinson & Tuovinen, 1984). Except for Hg^{2+} -resistant strains of *Enterobacter aerogenes* (Pan-Hou *et al.*, 1981) and *Clostridium cochlearium* (Pan-Hou & Imura, 1981), all other known cases of mercuric resistance have been associated with the presence of an intracellular mercury volatilizing enzyme, Hg^{2+} reductase (summarized in Summers & Silver, 1978; and Robinson & Tuovinen, 1984).

We report here the initial characterization of mercury volatilizing activities from two new groups of bacteria, *Streptomyces* species and group B *Streptococcus* from clinical sources. The properties of the Hg^{2+} reductase enzymes from these two new sources are similar to those previously reported from other Gram-positive bacteria (Weiss *et al.*, 1977; Izaki 1981; Olson *et al.*, 1982).

METHODS

Organisms. These are listed in Table 1.

Streptomyces species strains 5 and 8 were provided by Dr Shiau-Ta Chung, Upjohn Company, Kalamazoo, Michigan, USA, in the form of frozen mycelial pastes of cells grown in S medium (Hopwood *et al.*, 1977) at 32°C in the presence of 50 μM - Hg^{2+} . Strain 8 is *Streptomyces lividans* strain 66 from the Central Institute for Fermentation Microbiology in the German Democratic Republic. Strain 5 is a *Streptomyces spinosus* isolate. *Streptomyces lividans* strain 1326 (Bibb & Hopwood, 1981) was obtained from Professor David Hopwood's laboratory at the John Innes Institute, Norwich, UK. A spontaneous chloramphenicol-sensitive derivative of strain 1326, designated M252, was isolated by Schottel *et al.* (1981). *Streptomyces coelicolor* strains M130, M110, and M124 (Bibb & Hopwood, 1981) were also obtained from the John Innes Institute collection (Table 1). The *Streptomyces* strains not supplied as cell pastes were grown in liquid in yeast extract/malt extract medium (Bibb *et al.*, 1977) with added 34% (w/v) sucrose and 10 mM-MgCl₂ to an OD₆₅₀ of about 0.5. After addition of 1 μM - Hg^{2+} (for induction)

Table 1. *Bacterial strains and plasmids*

Organism	Strain	Chromosomal markers	Plasmids	Mercury resistance†	Reference
<i>Streptomyces coelicolor</i>	M130	<i>hisA1 uraA1 strA1</i>	None	R	Bibb & Hopwood (1981)
	M110	<i>hisA1 uraA1 strA1</i>	SCP2*	S	
	M124	<i>proA1 argA1 cysD18</i>	None	SS	
<i>Streptomyces lividans</i>	1326		SLP2, SLP3	R	Hopwood <i>et al.</i> (1983)
	M252		Not known	S	Schottel <i>et al.</i> (1981)
<i>E. coli</i>	C600(pDU1003)‡			R	NiBhriain <i>et al.</i> (1983)
<i>P. aeruginosa</i>	PAO 9501(pVS1)§			R	Clark <i>et al.</i> (1977)
<i>Staphylococcus aureus</i>	RN4		pI258	R	Novick & Roth (1968); Weiss <i>et al.</i> (1977)
<i>Streptococcus agalactiae</i>	B-14		Not known	R¶	Yamada <i>et al.</i> (1984)
	B-46		Not known	R	
	B-60		Not known	R††	

† R, Resistant; S, sensitive; SS, 'supersensitive'.

‡ Includes the R100 mercury resistance determinant.

§ Includes the Tn501 mercury resistance determinant.

¶ Additionally resistant to cadmium, arsenic and tetracycline.

|| Additional resistances as for B-14, but also chloramphenicol resistant.

†† Additional resistances as for B-14.

and an additional 6 h incubation, the mycelia were harvested by centrifugation and frozen. Hg²⁺ resistance of *Streptomyces* strains was tested by a disc assay (Weiss *et al.*, 1977) on plates containing yeast extract/malt extract medium with 10 mM-MgCl₂ plus 15 g agar l⁻¹ but without sucrose.

Hg²⁺-resistant strains of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Table 1) were used as controls in the Hg²⁺ reductase assays. These strains were grown and induced as previously described (NiBhriain *et al.*, 1983; Clark *et al.*, 1977; and Weiss *et al.*, 1977, respectively).

The *Streptococcus agalactiae* (Lancefield group B) strains (154 in number) were isolated from clinical specimens at the Juntendo University Hospital, Japan. The streptococci were grown in standing cultures in Todd-Hewitt Medium (Difco). Resistance to mercury was tested by a disc assay in Todd-Hewitt medium solidified with 15 g Bacto-agar l⁻¹.

Preparation of crude cell extracts. *Streptomyces* mycelia and cell pastes of *P. aeruginosa* and *E. coli* were stored frozen. Frozen cells were thawed and disrupted by two passages through a French pressure cell at 16000 lbf in⁻² (110 MPa). Streptococcal cells (at about 2 mg dry weight ml⁻¹) were disrupted in early experiments by addition of 10 mg lysozyme ml⁻¹, two cycles of freezing (dry ice/acetone bath) and thawing, and two passages through the French pressure cell. In later experiments, mutanolysin (Sigma) was used to lyse the streptococci. Lysostaphin (Sigma) was used to lyse *Staphylococcus aureus* cells (Weiss *et al.*, 1977).

Crude cell lysates of each organism were cleared by centrifugation (12000 r.p.m. for 2 to 5 min) in an Eppendorf centrifuge. The cell-free supernatant fluids, which contained almost all of the enzyme activity, were used as crude cell extracts.

Hg²⁺ reductase activity. This was measured as the disappearance of radioactive ²⁰³Hg from an assay mixture containing 50 mM-sodium phosphate buffer (pH 7.4), 0.2 mM-EDTA, 1 mM-β-mercaptoethanol and reduced pyridine nucleotide as described by Schottel (1978) and specified in the text. The 200 µl assay mixtures contained 25 µl enzyme sample (generally about 15 µg total protein) plus usually 5 to 10 µM ²⁰³Hg²⁺ (1.2 × 10⁵ to 4 × 10⁵ c.p.m. ml⁻¹). Samples of 25 µl were removed periodically during incubation at 37 °C with rapid (200 r.p.m.) shaking. The reactions were stopped by pipetting the samples directly into a water-miscible scintillation counting fluid, and the remaining radioactivity was counted by liquid scintillation spectroscopy.

RESULTS

Streptomyces strains

Streptomyces espinosus strain 5 and *Streptomyces lividans* strain 8 showed mercury resistance (S.-T. Chung, personal communication). Disrupted frozen mycelia of these strains grown in the presence of 50 µM-Hg²⁺ demonstrated Hg²⁺ reductase activity whereas cells grown in the absence of Hg²⁺ did not have such activity.

Fig. 1. Resistance of *Streptomyces* strains to HgCl₂ and Hg²⁺ reductase activity of crude cell extracts. (a) Zone of growth inhibition (mm diameter minus 6.5 mm of disc) with *Streptomyces lividans* strains 1326 (●, resistant) and M252 (○, sensitive), and *Streptomyces coelicolor* strains M130 (▲, resistant) or M110 (△, sensitive). (b) Volatilization of ²⁰³Hg by crude cell extracts of *Streptomyces lividans* strain 1326 (●, uninduced; or ○, induced by growth with 1 μM-HgCl₂) and strain M252 (▲, uninduced; or △, induced by growth on 1 μM-HgCl₂) at 37 °C. The complete assay mixture with 200 μM-NADPH was used.

Streptomyces lividans strain 1326 was found to be resistant to Hg²⁺ by a disc assay with 5 to 200 nmol HgCl₂ per disc. M252, a spontaneous chloramphenicol-sensitive mutant of strain 1326, was sensitive to Hg²⁺ (Fig. 1a). Strain 1326 showed Hg²⁺ reductase activity when grown on Hg²⁺-containing medium; when it was grown without HgCl₂, about 15% of the activity seen with the mercury-grown cells was detected (Fig. 1b). These results indicated a low level of constitutive enzyme synthesis in strain 1326. Strain M252 did not show Hg²⁺ reductase activity whether grown with or without HgCl₂ (Fig. 1b).

Streptomyces coelicolor strain M130 was as resistant to HgCl₂ as strain 1326 (Fig. 1a). *Streptomyces coelicolor* strain M110 was as sensitive to HgCl₂ as strain M252, and *Streptomyces coelicolor* strain M124 had a super-sensitive phenotype, giving inhibition zones of more than 30 mm with the lowest amount of HgCl₂ tested (5 nmol). Strain M130 showed Hg²⁺ reductase activity which was similar whether the cells were grown in the presence or absence of mercury, suggesting constitutive synthesis of Hg²⁺ reductase. Neither strain M110 nor strain M124 showed mercury volatilization activity under induced or uninduced conditions. The results with both the *Streptomyces lividans* and the *Streptomyces coelicolor* strains supported the previously established correlation between resistance to HgCl₂ and the synthesis of a Hg²⁺ reductase activity.

The Hg²⁺ reductase activities of crude cell extracts of the mercury-resistant strains of *Streptomyces* showed a requirement for thiol compounds (mercaptoethanol) and stimulation by EDTA (data not shown). Like the enzyme determined by plasmid R100 in *E. coli*, the enzyme from *Streptomyces lividans* strain 8 showed less than 10% of maximum activity in the absence of added mercaptoethanol and functioned optimally with 0.5 or 1 mM-mercaptoethanol. These results were similar to the requirements of crude enzyme preparations from *E. coli* (Schottel, 1978) and *Thiobacillus ferrooxidans* (Olson *et al.*, 1982). The enzyme from *Streptomyces lividans* strain 8, however, functioned relatively better with NADH (Fig. 2) than did comparable preparations with enzyme determined by plasmid R100 in *E. coli* or by *Pseudomonas* transposon Tn501 (Misra *et al.*, 1984, 1985). A time course of volatilization of mercury with these enzyme preparations and 25 μM-NAD(P)H was reported by Silver & Misra (1984). The crude enzymes from *Streptomyces lividans* strain 1326 and *Streptomyces coelicolor* strain M130 showed approximately similar volatilization rates with 50 μM or higher NADH and NADPH. In the range 10 μM to 25 μM-NAD(P)H, NADPH stimulated Hg²⁺ reductase activity more effectively than did NADH (data not shown).

Whereas the Hg²⁺ reductase enzymes from Gram-negative bacteria are generally very heat resistant and can withstand temperatures up to 80 °C, the comparable enzymes from Gram-positive bacteria tend to be about 20 °C less heat resistant (S. Silver & T. G. Kinscherf,

Fig. 2. Dependence on reduced pyridine nucleotide of Hg^{2+} reductase activity in crude cell extracts of (a) *E. coli* (with plasmid pDU1003, which contains the R100 system), (b) *P. aeruginosa* (with transposon Tn501), and (c) *Streptomyces lividans* strain 8. The assay mixture was prepared as described by Schottel (1978) with $10 \mu\text{M}$ - $^{203}\text{Hg}^{2+}$ and NADH (●) or NADPH (○) as indicated. The relative activities were determined from rates of volatilization of ^{203}Hg at 37°C . The 100% values for R100, Tn501 and *Streptomyces lividans* strain 8 were 41.4 , 39.9 and $2.8 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ respectively.

Fig. 3. Heat sensitivity of Hg^{2+} reductase activities. Crude cell extracts (0.2 ml) were heated for 10 min at the indicated temperature, then chilled to 4°C , and residual enzyme activity was assayed at 37°C with $10 \mu\text{M}$ - $^{203}\text{Hg}^{2+}$ and $200 \mu\text{M}$ -NADPH. The 100% values were set to those with enzyme incubated at 54°C . ○, *E. coli* carrying plasmid pDU1003 with the R100 system; ●, *P. aeruginosa* carrying Tn501; △, *Streptomyces lividans* strain 8; ▲, *Staphylococcus aureus* carrying pI258.

unpublished data; Fig. 3). In this regard, the enzymes from *Streptomyces lividans* strains 8 and 1326 and *Streptomyces coelicolor* M130 were very similar to that encoded by *Staphylococcus aureus* plasmid pI258 in strain RN4 (Weiss *et al.*, 1977). Mixing experiments with heat-sensitive and heat-resistant enzyme preparations have shown that the heat sensitivity or resistance is an inherent property of the enzyme and is not affected by other components of the crude enzyme mixture. Antiserum against enzyme encoded by Gram-negative plasmid R831 (Schottel, 1978) completely inactivated the enzyme encoded by plasmid R100, but it was without effect on the *Streptomyces lividans* strain 8 enzyme.

Group B *Streptococcus*

When screening clinical *Streptococcus* isolates, Yamada *et al.* (1984, and additional data) found 48 (out of a total of 154) isolates of *Streptococcus agalactiae* (group B *Streptococcus*) that showed resistance to Hg^{2+} salts; some were additionally resistant to antibiotics and/or cadmium and/or arsenic. The Hg^{2+} -sensitive strains showed a minimal inhibitory concentration of 37 or $74 \mu\text{M}$ - HgCl_2 in Todd-Hewitt broth agar, whereas the resistant isolates showed minimum inhibitory concentrations of 184 or $221 \mu\text{M}$ - HgCl_2 . This clear bimodal distribution of *Streptococcus agalactiae* into sensitive and resistant strains was similar to the bimodal distributions found earlier with other species (Nakahara *et al.*, 1977a, b). Strains B-14, B-46, and B-60 (Table 1) were selected from among the Hg^{2+} -resistant *Streptococcus agalactiae* isolates for further study.

Fig. 4

Fig. 4. Induction of Hg²⁺ reductase activity by *Streptococcus agalactiae*. Exponential phase cells were induced by exposure to 10 μM-Hg²⁺ for 3 h, during which time the cells increased threefold in number. The cells were harvested, resuspended at 25 mg dry weight ml⁻¹, and diluted ninefold into assay mixture containing 10 μM-Hg²⁺. Incubation was at 37 °C. ○, B-60 uninduced; ●, B-60 induced; △, B-14 uninduced; ▲, B-14 induced.

Fig. 5

Fig. 5. Pyridine nucleotide dependency of mercury volatilization by crude cell extracts of *Streptococcus agalactiae*. Conditions as in Fig. 2 legend. ○, B-46 with NADPH; ●, B-46 with NADH; △, B-60 with NADPH; ▲, B-60 with NADH. The 100% values were 145 and 184 nmol min⁻¹ per mg dry cell weight equivalent for strains B-46 and B-60 respectively.

Fig. 6. Heat sensitivity of Hg²⁺ reductase activities in crude cell extracts of *Streptococcus agalactiae*. Conditions as in Fig. 3 legend. The 100% values were set for those samples preincubated at 50 °C, and were measured as nmol min⁻¹ per mg cell dry weight equivalent. ○, *E. coli* carrying plasmid pDU1003 with the R100 system (100% = 1104); ●, *Staphylococcus aureus* carrying pI258 (100% = 79); △, *Streptococcus agalactiae* B-46 (100% = 153); ▲, *Streptococcus agalactiae* B-60 (100% = 145).

As with other Hg²⁺-resistant bacteria, the Hg²⁺ reductase system of the *Streptococcus* strains was inducible: strains B-14 and B-60 grown in the presence of Hg²⁺ showed Hg²⁺ reductase activity at the whole-cell level (Fig. 4) and in crude cell extracts (data not shown), whereas cells grown in the absence of Hg²⁺ did not show such activity (Fig. 4). With the crude cell extracts from strains B-46 and B-60, NADPH supported a higher level of enzyme activity than did NADH (Fig. 5). The use of NADH, however, resulted in more activity than seen in analogous experiments with the Hg²⁺ reductase from *E. coli* (Schottel, 1978; Fig. 2a of this paper). The enzymes from *Streptococcus agalactiae* strains B-46 and B-60 were only slightly more heat resistant than the *Staphylococcus aureus* enzyme and about 20 °C less heat resistant than the enzyme encoded by plasmid R100 in *E. coli* (Fig. 6).

DISCUSSION

The Hg^{2+} resistance system is among the most widespread of plasmid resistance systems both in Gram-negative and in Gram-positive bacteria (see Introduction). It is also one of the most thoroughly understood systems, both in terms of biochemistry (Fox & Walsh, 1982, 1983) and in terms of molecular genetics (NíBhriain *et al.*, 1983; Brown *et al.*, 1982; Misra *et al.*, 1984, 1985). With such background information, it is useful to extend the range of organisms for which this system has been reported. It is also a very useful system of plasmid resistance for species such as *Streptomyces* and *Streptococcus* in which people are trying to establish plasmid vectors with useful selective markers.

We have characterized the Hg^{2+} resistance systems in strains of *Streptococcus agalactiae*, *Streptomyces lividans* and *Streptomyces coelicolor*. Although the organisms used in this work are very different, the enzyme activities identified have characteristics similar to those previously associated with the Hg^{2+} reductase enzyme from other Gram-positive bacteria (Weiss *et al.*, 1977, 1978; Izaki, 1981; Silver & Kinscherf, 1982). Plasmids have been identified in *Streptomyces lividans* strain 8 (S.-T. Chung, personal communication), and strain 1326 (Hopwood *et al.*, 1983), and in *Streptomyces coelicolor* strain M110 (Bibb & Hopwood, 1981). There is no evidence, however, that these *Streptomyces* plasmids contain the Hg^{2+} resistance genes. Plasmids have not been identified as yet in the *Streptococcus agalactiae* strains. By comparison with earlier studies, the Hg^{2+} resistance systems of the strains we studied are likely to be governed by genes on plasmids or in transposon sequences, but as yet we know nothing about their location.

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