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Plasmid-Mediated Degradation of *O*-Phthalate and Salicylate by a *Moraxella* sp.

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A *Moraxella* sp. strain VG45 capable of utilizing *o*-phthalate and salicylate as a sole source of carbon and energy was isolated. The degradation of *o*-phthalate occurs via phthalate 4,5-dioxygenase, 4,5-dihydro-4,5-dihydroxyphthalate dehydrogenase, 4,5-dihydroxyphthalate decarboxylase and protocatechuate 4,5-dioxygenase. Salicylate is degraded via salicylate 5-hydroxylase, gentisate 1,2-dioxygenase and then by a glutathione-independent maleylpyruvate hydrolase. Further, a plasmid of app. 60 kilobase pairs (kb) is involved in the degradation of *o*-phthalate and salicylate and the enzymes of these two pathways are independently regulated in strain VG45.1996 Academic Press, Inc.

One of the important contributions of microorganisms to the environment is the degradation/mineralization of different organic compounds many of which have been identified as pollutants. Two groups of organic molecules, *o*-phthalate and salicylate commonly occur in the environment. *o*-Phthalate/phthalate esters are synthesized in plastics and textile industries, and their possible environmental pollution and toxicity to living organisms is of concern (10). Salicylate is a key intermediate in the catabolism of naphthalene by microorganisms (6). There are reports in the literature of microorganisms capable of utilizing *o*-phthalate or salicylate as sole source of carbon and energy, and in some cases, the genes for their degradation are plasmid-encoded (3,6,13,15,16,21,24). In this communication we report the isolation of a *Moraxella* sp. capable of utilizing both *o*-phthalate and salicylate as sole source of carbon and energy. Our results also demonstrate the involvement of a large plasmid of app. 60 kilobase pairs (kb) in their degradation. To our knowledge, this is the first report demonstrating the plasmid-encoded degradation of these compounds by a single organism.

MATERIALS AND METHODS

About 50 organisms, isolated from oil-field soil samples, were checked for their ability to grow on different aromatic compounds. One organism, designated as VG45, was found to be capable of utilizing *o*-phthalate, salicylate and gentisate as sole source of carbon and energy. However, this organism failed to utilize phenanthrene or naphthalene. The minimal medium (MM) of following composition was used for growth of strain VG45 (per litre)(9): Na_2HPO_4, 2.0g; KH_2PO_4, 1.0g; $(NH_4)_2SO_4$, 0.4g; $MgSO_4.7H_2O$, 0.4g; trace element solution, 2.0 ml. The trace element solution contained (per litre): $Al(OH)_3$, 0.1g; $SnCl_2.2H_2O$, 0.05g; KI, 0.05g; LiCl, 0.05g; $MnSO_4.4H_2O$, 0.08g; H_3BO_3, 0.05g; $ZnSO_4.7H_2O$, 0.1g; $CoCl_2.6H_2O$, 0.1g; $NiSO_4.6H_2O$, 0.1g; $BaCl_2$, 0.05g; $(NH_4)6Mo_7O_{24}.4H_2O$, 0.05g. The pH of the medium was adjusted to 7.4 and agarose (1.5%) was added for solidification of the medium. *o*-Phthalate and salicylate were added to a final concentration of 2 mM before autoclaving the medium. Gentisate was added as the filter sterilized solution (2 mM) after autoclaving the medium. The identification of strain VG45 was carried out by biochemical tests using Bergeys Manual of Systematic Bacteriology (Vol. 1, 1984)

For biochemical and enzymatic studies, strain VG45 was grown overnight in MM containing succinate (10mM) or one of either *o*-phthalate, salicylate, gentisate or protocatechuate (2mM). Methods of cultivation, conditions of growth and preparation of cell extracts were as described earlier (8). Protocatechuate 3,4-dioxygenase (EC 1.13.11.3) and protocatechuate 4,5-dioxygenase (EC 1.13.11.8) were assayed using crude cell- extracts essentially as described by Ono *et al.* (19). Salicylate hydroxylase (EC 1.14.13.1) was assayed according to the method of White-Stevens and Cumin (23) whereas

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catechol 1,2-dioxygenase (EC 1.13.11.1) and catechol 2,3-dioxygenase (EC 1.13.11.2) were assayed as described previously (14). The activities of gentisate 1,2-dioxygenase (EC 1.13.11.4) and salicylate 5-hydroxylase were determined as described by Crawford *et al.* (5) and Grund *et al.* (7), respectively. The activity of glutathione-dependent or glutathione-independent reaction was determined using N-ethylamaleimide in the reaction mixture (4,7). The activities of phthalate 4,5-dioxygenase and 4,5-dihydro-4,5 dihydroxyphthalate dehydrogenase were detected by the visual plate method described by Nomura *et al.* (15). This method is based on the diazo-coupling the reaction of a product from quinolinic acid by the action of the above enzymes.

Two methods were employed to isolate derivatives of strain VG45 incapable of growth on *o*-phthalate (OPA^-) or salicylate (SAL^-) or both. In the first method, spontaneous loss of the above phenotype(s) was tested by growth of strain VG45 in nutrient broth for 5 cycles followed by the inoculation of the culture(s) on nutrient agar plates. The single colonies thus obtained were checked by picking and patching onto MM agar plates containing either *o*-phthalate or salicylate as sole carbon source. In the second method, strain VG45 was grown in nutrient broth in the presence of ethidium bromide (10 μg/ml) which is a known curing agent for plasmids (12). The organism was grown for 5 cycles and single colonies were checked as indicated above. Plasmid DNA isolation in different strains was carried at according to the methods described previously (1,2,20,22). The plasmid DNA isolated by the method of Anderson and McKay (1) was used for restriction endonuclease digestion without any further purification. The conditions for restriction endonuclease digestion and electrophoresis were as described by Jain et al. (8). The conjugation experiments were performed using plate or filter matings as described earlier (8).

RESULTS AND DISCUSSION

Strain VG45 was found to be gram-ve, very short rods in shape, aerobic, oxidase positive, catalase positive, urease negative and non motile. No acid was produced from glucose, no indole was produced, gelatin was not liquefied, nitrate or nitrite were not reduced and no growth was observed at 5°C or in the presence of 6% NaCl. On the basis of the above characters, strain VG45 was identified as a *Moraxella* sp.

It is well-documented that the pathways for the catabolism of various aromatic hydrocarbons are often specified by the plasmids. Attempts were made to isolate the plasmid(s) from strain VG45 in order to check if the degradation of *o*-phthalate and/or salicylate is plasmid-encoded. Initial attempts to isolate plasmid(s) were failed using different methods (2,20,22). However, a large plasmid was detected when the method of Anderson and McKay (1) was followed (Fig. 1); the size of this plasmid was estimated to be app. 60 kb on the basis of the molecular weight marker plasmids

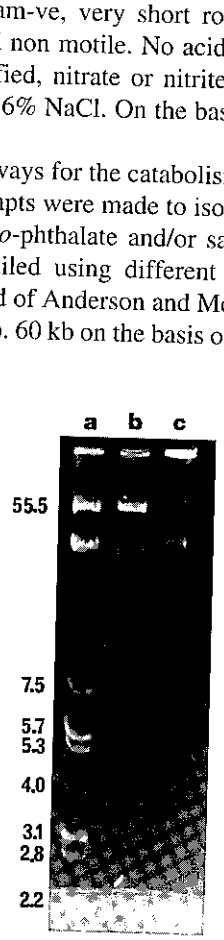

FIG. 1. Agarose gel electrophoresis of plasmid(s) isolated from *Moraxella* sp. strain VG45 (sizes shown in kb): Lanes **a,** *Escherichia coli* V517 as standard plasmid size markers; **b,** strain VG45; **c,** ethidium bromide-derived OPA^- SAL^- derivative of strain VG45. CHR, chromosome.

...nt in *E. coli* strain V517 (11; Fig. 1). The size of the plasmid was further confirmed following ...restriction endonuclease digestion by enzymes *Cla*I and *Hind*III (data not shown).

In order to determine the role of the plasmid present in strain VG45, attempts were made to ...ate derivatives incapable of growth on *o*-phthalate or salicylate or both. There was no sponta... loss of any of the above phenotype(s) when a total of 500 colonies were checked. However, ...en ethidium bromide was used as a curing agent, the frequency of loss of the OPA^+ SAL^+ ...enotype was app. 2%. A total of 9 derivatives out of 500 colonies tested failed to utilize either ...phthalate or salicylate as sole carbon source. No derivatives were obtained having the phenotype ...PA^+ SAL^- or OPA^- SAL^+. No plasmid(s) was detected in any of the OPA^- SAL^- derivatives ...ed by any of the methods described above (1,2,20,22). This suggested that the plasmid present ...strain VG45 is involved in *o*-phthalate and salicylate degradation. Further, our results also ...gested that this plasmid is nonconjugative since we were unable to transfer the plasmid by plate ...filter matings to other plasmid-free *Pseudomonas putida* strains (data not shown).

To determine the pathways for *o*-phthalate and salicylate degradation and the role of the plasmid ...their degradation, enzyme analyses of the above pathways was carried out. It has been reported ...at the degradation of *o*-phthalate occurs via the formation of protocatechuate which is further ...metabolized via either 3,4-dioxygenase (*ortho*-cleavage) or 4,5-dioxygenase (*mela*-cleavage) en... (6,17,18). In strain VG45, activities of phthalate 4,5-dioxygenase and 4,5-dihydro-4,5 dihy...oxyphthalate dehydrogenase were detected as indicated by the visual plate method (please see ...bove); these two enzymes are involved in the conversion of *o*-phthalate into protocatechuate. In ...order to determine the further oxidation of protocatechuate, protocatechuate 3,4-dioxygenase and ...protocatechuate 4,5-dioxygenase enzymes were assayed using succinate-grown cells (for consti...tutive activities) and *o*-phthalate-grown cells (for inducible activities) of strain VG45. No activity ...of protocatechuate 3,4-dioxygenase was detected when strain VG45 was grown either on succinate ...or on *o*-phthalate. However, an activity of protocatechuate 4,5-dioxygenase was present when the ...organism was grown in the presence of *o*-phthalate (120 nm substrate utilized/min/mg protein); no ...constitutive activity of this enzyme was detected. The above results clearly indicated that the ...degradation of *o*-phthalate in strain VG45 occurs via protocatechuate which is further metabolized ...by protocatechuate 4,5-dioxygenase enzyme. This pathway is shown in Fig. 2. When ethidium ...bromide-cured, OPA^-SAL^- derivative of strain VG45 was grown on succinate or on succinate ...containing *o*-phthalate or protocatechuate, no activity of protocatechuate 4,5-dioxygenase was ...detected. Furthermore, this derivative strain also failed to show any phthalate 4,5-dioxygenase and ...4,5-dihydro-4,5-dihydroxyphthalate dihydrogenase activities. These results, therefore, clearly in...dicated that the plasmid present in strain VG45 is involved in *o*-phthalate degradation.

The degradation of salicylate by bacteria mainly occurs via either *ortho*- or *meta*-cleavage ...pathway through catechol formation (6,24,25). There are only two reports which show that the ...metabolism of salicylate occurs via gentisate (7,13). We have determined the pathway for salicylate ...degradation in strain VG45. No activities of salicylate hydroxylase and catechol 1,2-dioxygenase ...or catechol 2,3-dioxygenase were obtained when strain VG45 was grown either on succinate or on ...salicylate. This showed that salicylate is not degraded via the formation of catechol. On the ...other hand, the activities of gentisate 1,2-dioxygenase and salicylate 5-hydroxylase were detected ...when this organism was grown on salicylate. These results are shown in Table 1. This, therefore, ...showed that salicylate is metabolized through gentisate which is further oxidized by gentisate ...1,2-dioxygenase (Fig. 2). Preliminary studies were also carried out to check whether the metabo...lism of maleylpyruvate, formed from gentisate by the action of gentisate 1,2-dioxygenase, occurs ...via a glutathione-dependent or glutathione-independent reaction (4). When strain VG45 was grown ...on salicylate, the maleylpyruvate produced in cell extracts of the organism disappeared in a ...glutathione-independent reaction; this was concluded by the fact that the decrease in the absorbance ...(A_{334}) in the reaction mixture could not be inhibited by the addition of N-ethylmaleimide (4,7).

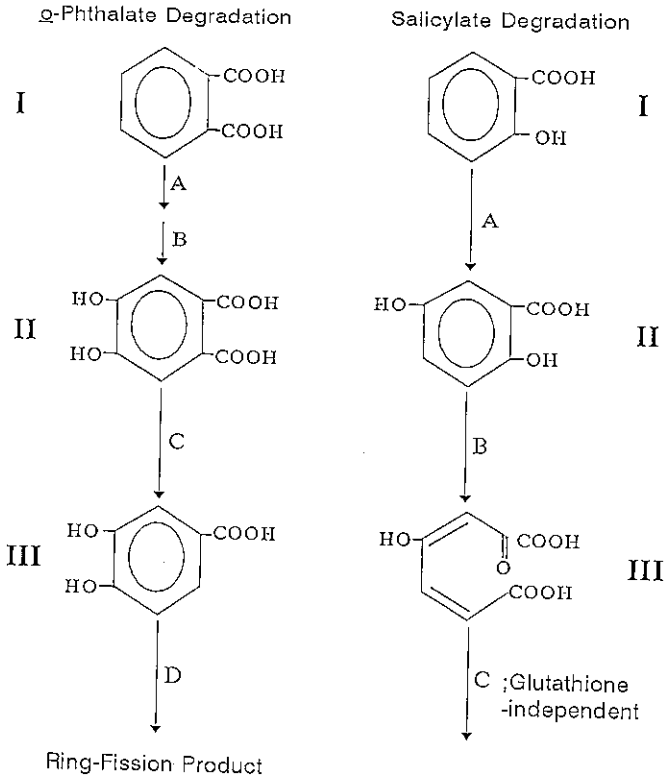

FIG. 2. Pathways for the degradation of *o*-phthalate and salicylate by *Moraxella* sp. strain VG45. *o*-Phthalate degradation—*Compounds shown are:* I, o-phthalate; II, 4,5-dihydroxyphthalate; III, protocatechuate. *Enzymes shown are:* A, phthalate 4,5-dioxygenase; B, 4,5-dihydro-4,5-dihydroxyphthalate dehydrogenase; C, 4,5-dihydroxyphthalate decarboxylase; D, protocatechuate 4,5-dioxygenase. Salicylate degradation — *Compounds shown are:* I, salicylate; II, gentisate; III, maleylpyruvate. *Enzymes shown are:* A, salicylate 5-hydroxylase; B, gentisate 1,2-dioxygenase; C, maleylpyruvate hydrolase.

When ethidium bromide-cured, OPA^- SAL^- derivative of strain VG45 was grown on succinate containing salicylate or gentisate, no activities of salicylate 5-hydroxylase and gentisate 1,2-dioxygenase were detected. This demonstrates that the plasmid present in strain VG45 is involved in salicylate degradation.

We further wanted to establish if there was any correlation between the enzymes of the two pathways described above. No activities of salicylate 5-hydroxylase and gentisate 1,2-dioxygenase enzymes were detected when strain VG45 was grown on *o*-phthalate. Similarly, cells grown on

TABLE I
Specific Activities of Enzymes of Salicylate-Degrading Pathway in *Moraxella* sp. Strain VG45

	Specific activities[a]	
Carbon Source	Salicylate 5-hydroxylase	Gentisate 1,2-dioxygenase
Succinate	<1.0	<1.0
Succinate + Salicylate	22	82

[a] The specific activities are expressed as moles of substrate utilized or product formed/min/mg protein. Data shown are the mean of values obtained from two different experiments.

salicylate did not show the activities of phthalate 4,5-dioxygenase, 4,5-dihydro-4,5-dihydroxyphthalate dehydrogenase, protocatechuate 3,4-dioxygenase or protocatechuate 4,5-dioxygenase. This indicated that these two pathways are independently regulated and that the genes encoding these pathways are likely to be located separately on the plasmid present in strain VG45.

The results presented in the above work show that an app. 60 kb plasmid, present in *Moraxella* sp. strain VG45, is involved in the degradation of *o*-phthalate and salicylate. This is concluded by the fact that the ethidium bromide cured, OPA^- SAL^- derivative strain was devoid of any plasmid and that no activities of any of the enzymes of the above two pathways were detected. However, this is the subject of further investigation if all the regulatory/structural genes encoding above enzymes are located on the plasmid. One of the approaches to a more detailed analysis of the regulation of synthesis of these enzymes is to clone these genes from strain VG45. Based on the enzyme analyses of the two pathways it is clear that *o*-phthalate and salicylate are degraded by different sets of enzymes and are separately controlled. To our knowledge, this is the first report which shows the involvement of a plasmid in the degradation of both the compounds, *o*-phthalate and salicylate, simultaneously by a single organism. Such organism(s) may prove to be useful for the purpose of decontamination of the pollutants in the environment.

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