Bacterial Metabolism of Arylsulfonates

1. Benzene Sulfonate as Growth Substrate for *Pseudomonas testosteroni* H-8[1]

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Pseudomonas testosteroni H-8 utilizes as sole carbon source benzene sulfonate (BS), *p*-toluene sulfonate (pTS), and ethylbenzene sulfonate (EBS) but not higher homologs. Growth on BS was rapid (generation time, 3 hr) and efficient ($Y = 57$), and resulted in accumulation of sulfate. As the culture is acid-sensitive, the medium must be heavily buffered to permit extensive growth. The BS oxidase system is inducible. Cells grown on BS, but not glutamate, oxidized BS, pTS, or EBS without lag ($QO_2 = 50$ to 100). Oxygen uptake on BS is temperature-dependent and sensitive to cyanide. Complete oxidation of 1 μmole of BS consumed approximately 5.7 μmoles of oxygen.

The degradation of detergents in nature by bacteria is an interesting problem both theoretically and practically. Biodegradable detergents of commerce are primarily of the linear alkylbenzene sulfonate (LAS) class and consist of a straight-chain alkyl portion and an arylsulfonate moiety. Such *n*-alkylbenzene sulfonic acids constitute an homologous series of compounds, the lower members of which lack detergent properties but are readily obtained as single isomers. Bacteria initially oxidize the hydrocarbon chain of LAS detergents by β oxidation, which has been well studied, but less is known about the subsequent attack on the aromatic portion (12).

Because of certain practical difficulties in the use of the LAS detergents themselves, we have attempted to learn more about the dissimilation of arylsulfonates in general by studying "model" compounds such as benzene sulfonate (BS) and *p*-toluene sulfonate (pTS). We have isolated and identified, as *Pseudomonas testosteroni* H-8, an organism which can utilize benzene sulfonate as the sole carbon, sulfur, and energy source. This report describes the growth of this organism on certain simple arylsulfonates and some observations regarding the oxidation of these compounds by washed cell suspensions.

MATERIALS AND METHODS

Bacterial cultures and growth media. A bacterium capable of extensive growth on BS was isolated from pond water and identified tentatively as *P. testosteroni* H-8 by the methods of Stanier et al. (10). This cul-

ture was maintained on slants of mineral salts medium containing 1 g of BS per liter. The mineral salts medium was a modification of that of Leadbetter and Foster (7) and consisted of the following ingredients (per liter of distilled water): $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; KH_2PO_4, 3.0 g; K_2HPO_4, 7.0 g; $CaCl_2$, 10 mg; $FeSO_4 \cdot 7H_2O$, 5 mg; $MnSO_4$, 2 mg; $CuSO_4$, 50 μg; H_3BO_4, 10 μg; $ZnSO_4$, 70 μg; and MoO_3, 10 μg. This basal medium was supplemented with a suitable carbon source at the concentration of 1 g/liter, unless otherwise specified.

Conditions for shake flask experiments. Conical flasks with metal closures (DeLong flasks, Bellco Glass Co., Vineland, N.J.) received 50 ml of medium per 250-ml flask or 200 ml per 1,000-ml flask and were incubated with rotary shaking (250 rev/min) at 30 C in a water bath (Metabolyte shaker, New Brunswick Scientific Co., New Brunswick, N.J.). Growth was assessed by turbidity measurements at 600 nm with a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.), and correlated with dry weight by means of a previously constructed calibration curve.

Radioactivity measurements. ^{35}S-BS (15 mCi/mmole) was supplied to cultures in mineral salts medium at a level of 0.025 μCi/ml. Incorporation of radioactive sulfur into cellular polymers was determined by addition of 2 ml of ice-cold 10% trichloroacetic acid to 2 ml of culture. After 60 min at 0 C, acid-insoluble material was recovered by membrane filtration (0.45 μm; Millipore Corp., Bedford, Mass.). Analysis of BS and inorganic sulfate was based on barium solubility. Cells were removed from samples of culture by membrane filtration, and 2-ml samples of filtered broth were treated with 2 ml of 10% $BaCl_2$ solution and filtered to recover barium-precipitable material. The filters and samples of filtrates were dried in aluminum planchets, and radioactivity was determined with a gas-flow proportional counter

(Nuclear-Chicago Corp., Des Plaines, Ill.). No corrections were made for self-absorption.

Respiration of washed cell suspensions. Conventional manometric techniques (13) were used to measure respiration of twice-washed cell suspensions. Vessels received 6 to 10 mg (dry weight) of washed cells, 50 μmoles of phosphate buffer (pH 7.0), and various amounts of substrate in a volume of 2.8 ml. The center well received 0.2 ml of 20% KOH; oxygen consumption was measured at 30 C.

Miscellaneous analytical methods. Sulfite was determined by iodometric titration (8) and also by the Robinson test (9). Sulfate was determined turbidimetrically after $BaCl_2$ precipitation (2).

Chromatography of arylsulfonates. Compounds were applied to Whatman no. 1 filter paper and developed by ascending or descending chromatography by using one of the following solvent systems: (i) 1-butanol-acetic acid-water (8:2:2); (ii) methylethylketone-ethanol-ammonia (30:6:2); (iii) 1-butanol saturated with ammonia; (iv) 1-butanol-ethanol-water (4:1:5). Pinacryptol yellow (0.05% in water) spray was employed to locate arylsulfonates by fluorescence in ultraviolet light (3).

Chemicals. BS, toluene sulfonate, and p-hydroxybenzoate were obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. ^{35}S-BS (14 mCi/mM) was purchased from Tracerlab, Waltham, Mass. Catechol sulfonate (pyrocatechol sulfonic acid) was obtained as a special preparation (6) from Distillation Products Industries, Eastman Organic Chemicals, Rochester, N.Y. Dodecylbenzene sulfonate and pinacryptol yellow were obtained from K&K Laboratories, Plainview, N.Y.

Short-chain alkylbenzene sulfonates, namely the ethyl-, propyl-, butyl-, and hexylbenzene sulfonates, were prepared by sulfonation of the corresponding alkylbenzene with fuming sulfuric acid (1) and recrystallization from ethanol.

The following alkylbenzenes were obtained from Eastman Organic Chemicals, Rochester, N.Y.: ethylbenzene, propylbenzene, butylbenzene, and 1-phenylhexane (i.e., n-hexylbenzene).

The N-2,4-dinitroanilinomaleimide reagent for the Robinson sulfite test was prepared in the laboratory (5).

Other chemicals were obtained from commercial suppliers and were the highest purity available.

RESULTS

Survey of arylsulfonates utilized as growth substrates by P. testosteroni H-8. *P. testosteroni* H-8 was able to utilize BS as the sole source of carbon and sulfur for growth. Since it was of interest to determine the range of sulfonated substrates which can be used by this organism, 15 different sulfonated compounds (sodium salts) were tested as the sole carbon source (1 g/liter) for growth of this bacterium.

Included in this survey were members of the homologous series of n-alkyl substituted benzene sulfonic acid. Paper chromatography of these substances by using four solvent systems revealed single spots which gave a pink fluorescence with pinacryptol yellow. Of this series, H-8 was able to utilize BS, pTS, and ethylbenzene sulfonate (EBS). However, the higher homologs of this series, the propyl-, butyl-, hexyl-, and dodecylbenzene sulfonates, did not support growth. It was observed that the benzene sulfinic and p-toluene sulfinic acids also gave slight but definite growth.

The culture failed to grow with any of the other related compounds which were tested, including 2,5-dimethylbenzene sulfonate, 2,4-dimethylbenzene sulfonate, p-sulfobenzate, p-phenol sulfonate, and catechol sulfonate.

Paper chromatography of the catechol sulfonate sample with solvent (i) revealed three ferric chloride-reactive spots, with R_F values of 0.35, 0.16, and 0.01, respectively. None of these gave a fluorescent reaction product with pinacryptol yellow. No further efforts to ascertain the identity of these three compounds were made.

No growth occurred in control flasks of the mineral salts base alone without an added carbon source.

Growth kinetics. Several experiments were conducted to characterize the growth of *P. testosteroni* H-8 in mineral salts medium with BS as the source of carbon. Optimum growth was obtained when the culture was grown at 30 C with shaking and a neutral or slightly alkaline pH was carefully maintained. Maximum growth was reached within 48 hr; and, with saturating levels of BS (10 g/liter), this value was 1.3 to 1.5 optical density units (600 nm). With washed cells from an exponentially growing culture, it was found that an OD_{600} nm of 1.0 was equivalent to 0.49 mg (dry weight)/ml. Under the conditions employed, the mass doubling time of the culture during exponential growth, as indicated by turbidity measurements, was approximately 3 hr.

Formation of sulfate during growth on BS. During growth of H-8 on BS, the sulfonic acid group was removed and converted to sulfate, as indicated by an increase in barium chloride-precipitable material. Contrary to the results of Cain and Farr (4), the production of sulfate from BS by H-8 did not appear to involve accumulation of detectable amounts of sulfite. Several unsuccessful attempts were made to demonstrate sulfite in the culture medium during growth of H-8 on BS. Similarly, iodometric titrations and the Robinson test (10) failed to detect sulfite formation during oxidation of BS by dense washed cell suspensions.

A small amount of the sulfur from BS was incorporated into cellular constituents. As shown in Fig. 1, when ^{35}S-BS was supplied to the culture as the sole carbon and sulfur source, the radioactive substrate, which was not precipitated by
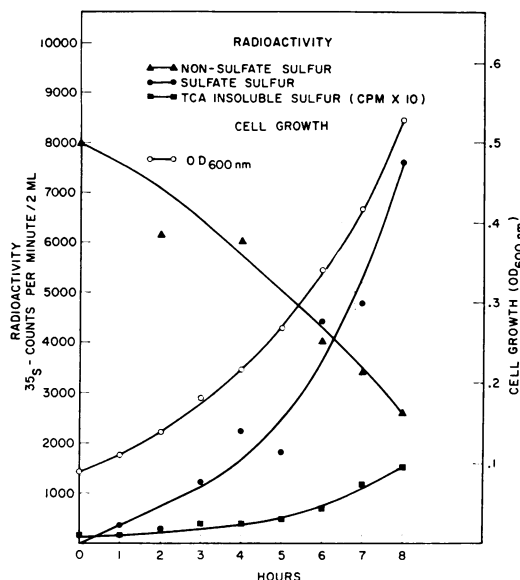
FIG. 1. *Production of* ^{35}*S-sulfate during growth of P. testosteroni H-8 on* ^{35}*S-labeled benzene sulfonate.* ^{35}*S-benzene sulfonate (25* μ*Ci/liter) was added to a culture growing in mineral salts medium with 1 g/l of nonradioactive benzene sulfonate per liter, and samples (2 ml) were removed at intervals for determination of barium-soluble (nonsulfate) and barium-insoluble (sulfate) radioactivity.*

BaCl$_2$, was progressively converted to BaCl$_2$-precipitable material. This material was presumably inorganic sulfate. At the same time, a small portion of the radioactivity was incorporated into the fraction of the cells which is insoluble in cold 5% trichloroacetic acid.

The conversion of BS to sulfate is accompanied by an increase in acidity, which can be of such magnitude as to limit growth. When *P. testosteroni* H-8 was grown in the mineral salts medium with 1 g of BS per liter, it was observed that a phosphate concentration of 0.06 M or more was sufficient to maintain reaction at *p*H 7.0 and give good growth. For this reason, the mineral salts medium adopted for subsequent experiments contained 0.06 M phosphate. The high level of phosphate was necessary only with arylsulfonates. Other growth substrates, such as glutamate or *p*-hydroxybenzoate, supported extensive growth even in a low-phosphate medium.

Growth yields with arylsulfonates as carbon source. Maximum growth attained was closely correlated with, and proportional to, BS concentration in the range 0 to 5 g/liter. Increasing the substrate concentration beyond 5 g/liter did not yield further increments in total growth. From the growth response to substrate under conditions of substrate limitation, growth yields could be calculated.

The molar growth yield coefficient (grams of dry weight of cells per mole of substrate utilized) for *P. testosteroni* H-8 on BS in well buffered medium was approximately 57. Contrary to the experience of Cain and Farr (4) with another species of *Pseudomonas*, growth of H-8 on BS was of the same order of magnitude as that obtained with other carbon sources. A comparison was made of the growth response of H-8 to various concentrations of several carbon sources. In Table 1 are shown the cell yields and molar growth yields calculated from the maximum growth under conditions where growth was limited by, and proportional to, the amount of carbon source supplied. Growth on BS was generally comparable to that on glutamate or *p*-hydroxybenzoate but somewhat less than on pTS.

Oxidation of arylsulfonates by washed cell suspensions. Washed cells prepared from cultures of *P. testosteroni* H-8 grown in mineral salts medium with BS as the carbon source were capable of rapid oxidation of BS, pTS, and EBS, but not the higher homologs. The QO$_2$ values for BS oxidation by such suspensions ranged from 45 to 145 but generally were around 85 to 95. A typical experiment is shown in Fig. 2, in which a QO$_2$ value of 72 was obtained. A determination of carbon dioxide evolution during the oxidation of BS indicated an RQ value of approximately 0.7.

When limiting amounts of BS were supplied, the oxidation went to completion. Cells incubated at 30 C with 2.0 μmoles of BS consumed 285 μliters of oxygen for completed oxidation (Fig. 2). This is equivalent to approximately 5.7 μmoles of O$_2$ per μmole of BS. The rate of oxidation of BS was temperature-dependent. Between 20 and 30 C, the Q$_{10}$ value was approximately 2.3.

Washed cell suspensions of H-8 were able to

TABLE 1. *Growth yield of P. testosteroni with arylsulfonates and with nonsulfonated carbon sources*

Carbon source[a]	Growth yield	
	Cell growth[b]	Molar growth yield (*Y*)[c]
Benzene sulfonate	0.31	57
p-Toluene sulfonate	0.42	82
p-Hydroxybenzoate	0.33	46
L-Glutamate	0.34	52

[a] As sodium salt.
[b] Gram of cells per gram of substrate.
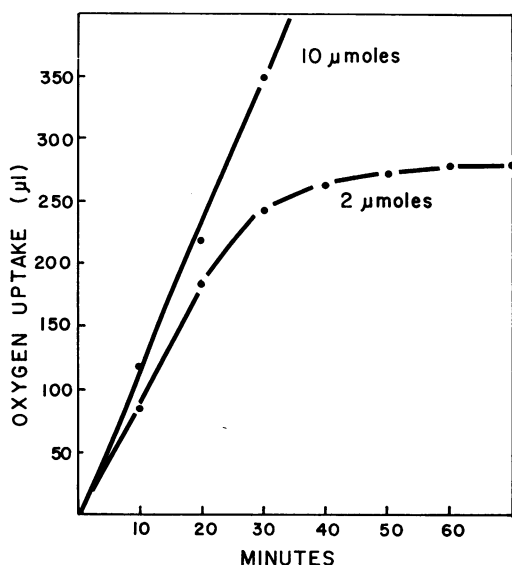[c] Gram of cells per mole of substrate.

FIG. 2. *Oxidation of benzene sulfonate by washed cells of P. testosteroni H-8 grown on benzene sulfonate. Warburg vessels received 7.5 mg (dry weight) of cells, 50 μmoles of phosphate buffer (pH 7.0), and indicated amount of benzene sulfonate.*

oxidize arylsulfonates rapidly only when these compounds had been present in the original growth medium. The QO_2 values for BS oxidation by cells grown on glutamate or p-hydroxybenzoate were very low, 5 and 7 respectively, whereas cells grown on BS exhibited a QO_2 of 93.

Similar manometric determinations were made on cells grown overnight in complex medium (nutrient broth) with and without added BS to observe the effects of readily utilizable nutrients on the induction of the arylsulfonate oxidation system. The results were similar to those in syn-

thetic medium. Cell suspensions could oxidize BS and pTS immediately without lag only when an arylsulfonate had been present in the growth medium (Fig. 3). It is clear from the results in Fig. 3 that the presence of other nutrients in the growth medium does not prevent the formation of the inducible arylsulfonate oxidation system.

The oxidation of BS by washed cell suspensions was markedly inhibited by potassium cyanide but was much less sensitive to sodium azide. A concentration of approximately 8×10^{-4} M KCN was required to produce a 50% inhibition of oxygen uptake, and increasing this to 10^{-2} M gave 85% inhibition.

Oxidation of BS was strongly inhibited by iodoacetate, N-ethylmaleimide, mercuric chloride, and sodium arsenite. No significant inhibition resulted from the addition of quinacrine, sodium fluoride, 2,4-dinitrophenol, ethylenediaminetetraacetic acid, hydroxylamine, or sodium bisulfite.

DISCUSSION

The ability of H-8 to oxidize BS or toluene sulfonate clearly has no necessary relationship to its capability of attacking the long-chain homologs. If the reaction sequence for toluene sulfonate proposed by Cain and Farr (4) correctly describes the pathway used by H-8, there is no direct attack on the alkyl group. The methyl group of toluene sulfonate is maintained unchanged through ring cleavage and finally becomes the terminal methyl group of propionaldehyde. However, in the case of LAS detergents, the long alkyl chains appear to be attacked initially (11, 12). Possibly this accounts for the inability of H-8 to grow on alkylbenzene sulfonates having more than a C_2 alkyl group; compounds having longer side-chains would have to be metabolized via another pathway unavailable to H-8.
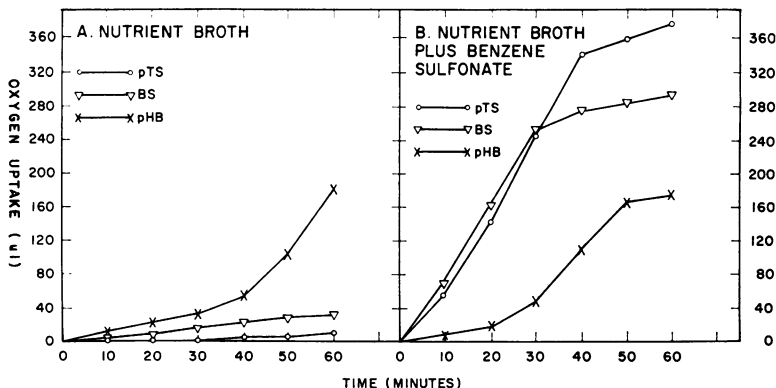

FIG. 3. *Failure of complex medium to prevent induction of benzene sulfonate (BS) oxidation system in P. testosteroni H-8. Respiration of washed cells prepared from cultures grown on nutrient broth (A) or nutrient broth supplemented with 1 g of BS per liter (B) was determined by using 2 μmoles of BS, p-toluene sulfonate (pTS), or p-hydroxybenzoate (pHB).*

Although we have considered BS in relation to detergents, BS is in itself an industrial waste product in some cases, and its disposal may present problems. We find that some, but not all, organisms present in sewage and river water can attack BS and that in H-8 the overall oxidation system for BS is inducible. Our results indicate that optimal biodegradation of BS would require an "adapted" bacterial flora and careful control of the acidity which results from sulfate formation.

Our results with H-8, although in general agreement with those of Cain and Farr (4) with *P. aeruginosa* strains, differ in at least two respects. First, we were able to obtain much more extensive growth of H-8 on BS than they reported with their cultures. It seems very likely that this was due to the greater buffering capacity of the culture medium which we have used. We believe this to be an important practical consideration and worthy of restatement: during biodegradation of arylsulfonates, bacterial growth can be limited by the accumulation of acid. Consequently, high levels of buffer or continuous neutralization must be employed to permit maximum growth.

A second point of difference is that we could not detect any accumulation of sulfite in the medium during the attack on BS. We suggest that this could be due to a difference in the sulfite-oxidizing capacity of the cultures. A culture with an active sulfite oxidase, such as ours, might fail to accumulate sulfite due to its rapid conversion to sulfate, whereas another culture lacking a sulfite oxidase would accumulate the compound.

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