Utilization of Primary and Secondary Alcohols by the Detergent-degrading Bacterium *Pseudomonas* C12B

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The capacity of the detergent-degrading bacterium *Pseudomonas* C12B to grow on, and oxidize, a range of primary and secondary alcohols of chain lengths from C₂ to C₁₆ was examined. The organism was able to grow on most primary alcohols tested, and especially well on the C₁₀–C₁₂ homologues. Of several secondary alcohols, C₁₁–C₁₃ homologues of alkan-2-ols supported good growth, as did some long-chain symmetrical or near symmetrical alcohols. There was constitutive synthesis of primary and alkan-2-ol dehydrogenase activities, enabling the organism to oxidize primary alcohols and alkan-2-ols up to at least C₁₁. Experiments with resolved D- and L-octan-2-ols suggested that the constitutive alkan-2-ol dehydrogenase activity was specific for D-isomers. Growth on secondary alcohols induced synthesis of L-alkan-2-ol dehydrogenase(s) and also greatly increased the capacity of extracts to oxidize racemic alkan-2-ols in the range C₆–C₁₁. An enzyme with high activity towards symmetrical alcohols was also induced by growth on secondary (especially symmetrical) alcohols. Collectively, the various alcohol dehydrogenase activities detected would enable *Pseudomonas* C12B to oxidize all the alcohols liberated from mixed alkyl sulphate detergents by the organism's complement of two primary and three secondary alkylsulphatases.

INTRODUCTION

Pseudomonas C12B, a detergent-degrading bacterium originally isolated from soil near a sewage outfall (Payne & Feisal, 1963), is able to degrade primary and secondary alkyl sulphate, alkanesulphonate and alkyltriethoxy sulphate surfactants. Degradation of the alkyl sulphates is initiated by the action of five alkylsulphatase enzymes (Dodgson & White, 1983). Two of these enzymes (designated P1 and P2) are active towards primary alkyl sulphates of chain length C₆ or greater (e.g. sodium dodecyl sulphate, SDS) whereas the remaining three enzymes (S1, S2 and S3) are secondary alkylsulphatases. The S1 and S2 enzymes are active towards D- and L-isomers, respectively, of alkan-2-sulphates of chain length C₆ or greater. The S3 enzyme is active towards symmetrical (e.g. nonan-5-sulphate) or near-symmetrical alkyl sulphates.

Primary alcohols liberated by the action of the P1 and P2 enzymes are considered (Dodgson & White, 1983; Williams *et al.*, 1966) to undergo oxidation via aldehydes to carboxylic acids which in turn serve as substrates for β -oxidation. Metabolism of secondary alcohols is more complicated, requiring C–C bond cleavage to produce substrates for β -oxidation (Lijmbach & Brinkhuis, 1973), but oxidation of the secondary hydroxyl groups to ketones is still the first step. *Pseudomonas* C12B can grow on, and oxidize, linear primary alcohols with even- and odd-numbered carbon-chains from C₂ to C₁₁ (Payne, 1963; Williams *et al.*, 1966). Moreover, cell extracts contained NAD-linked dehydrogenase(s) active towards these primary, and certain secondary, alcohols.

Studies on *Comamonas terrigena*, a co-isolate of *Pseudomonas* C12B and taxonomically related to *Pseudomonas testosteroni* and *Pseudomonas acidovorans* (Hugh, 1965; Stanier *et al.*, 1966; Palleroni *et al.*, 1973), have shown that this organism produces two alkylsulphatases only, one of

which (designated CS1) acts on L-alkan-2-sulphates of chain length C_6 or greater, and the other (CS2) on the corresponding D-isomers (Dodgson & White, 1983; Matcham *et al.*, 1977; Barrett *et al.*, 1980a). Like the S1, S2 and S3 secondary alkylsulphatases, the two *C. terrigena* enzymes operate by C–O bond fission with inversion of configuration at the esterified carbon atom, so that D-esters are converted to L-alkan-2-ols and vice versa. The sulphatase stereospecificity is matched by a pair of alcohol dehydrogenases, one of which acts on D-alkan-2-ols and primary alcohols, and the other on L-alkan-2-ols, symmetrical secondary alcohols and probably other positional isomers of secondary alcohols of the L-configuration (Barrett *et al.*, 1980b; Barrett *et al.*, 1981).

In view of the complementarity between alcohol dehydrogenases and alkylsulphatases in *C. terrigena*, the question now arises as to whether a similar situation obtains in *Pseudomonas* C12B. Because the latter organism has a more versatile complement of alkylsulphatases, as well as a much greater capacity for detergent degradation, it might be expected that a greater variety of alcohol dehydrogenases might also be present. The present work was undertaken to investigate the ability of *Pseudomonas* C12B to grow on a variety of primary and secondary alcohols and to attempt to relate this ability as far as possible to the alcohol dehydrogenase activities produced by the organism.

METHODS

Chemicals. Nutrient broth and agar were obtained from Difco. Primary and secondary alcohols for growth experiments and enzyme assays were obtained from Aldrich, BDH, Eastman, Fluka, ICN, K and K and Koch Light. SDS purchased from BDH was recrystallized from methanol before use. All other reagents were obtained from BDH or Sigma.

Organism and growth. The isolation of *Pseudomonas* C12B (NCIB 11753) has been described elsewhere (Payne & Feisal, 1963). The organism has been maintained (in this laboratory since 1972) by monthly transfers on nutrient agar containing 0.8% (w/v) nutrient broth solidified with 1.5% (w/v) agar. Basal salts medium was prepared as described by Fitzgerald & Payne (1972) with the addition of 0.014% Na_2SO_4 . Nutrient broth (0.8%) and basal salts alone or containing 1% (w/v) pyruvate or undecanoate were autoclaved whole, but all alcohols were added aseptically (0.1%) to autoclaved media. For growth experiments, 250 ml Erlenmeyer flasks containing 50 ml of medium were inoculated (0.02%) with a 24 h nutrient broth culture and shaken (100 r.p.m.) at 30 °C. After 24 h, culture optical densities were measured at 650 nm (broth) or 420 nm (defined media) in cuvettes of 1 cm light path in a Cecil CE292 spectrophotometer. Duplicate values greater than 0.1 were usually repeatable within $\pm 14\%$. Duplicates less than 0.1 were repeatable within ± 0.03 OD units.

Estimations. For measurements of enzyme activities, *Pseudomonas* C12B was grown in 1 litre Erlenmeyer flasks containing 400 ml of medium. Bacteria were harvested in late-exponential phase by centrifuging ($10000 g_{av}$, 15 min) and resuspended in approx. 5 ml 10 mM-Tris/HCl buffer, pH 7.5. Chilled cell suspensions were disrupted using a French pressure cell operating at 126 MPa (3 passes per sample) and cell debris was removed by centrifugation ($10000 g_{av}$, 1 h). Protein content of the cell extracts was measured by the method of Bradford (1976), and alcohol dehydrogenase activities were assayed using NAD^+ as co-factor and one of a variety of primary and secondary alcohols as described previously (Barrett *et al.*, 1980b). For duplicate growth experiments, enzyme assays were generally within $\pm 10\%$ of the mean.

RESULTS AND DISCUSSION

Growth experiments

Pseudomonas C12B was able to grow on a wide variety of alcohols (Fig. 1). In a 24 h incubation period with primary alcohols (Fig. 1a), growth was most extensive with the C_{10} – C_{12} group. No growth was apparent with nonan-1-ol within the 24 h growth period, but lower primary alcohols (C_6 – C_8) and the higher alcohol, tetradecan-1-ol, supported modest growth. As the carbon chain length was decreased below C_6 , or increased above C_{14} , growth became progressively more feeble. Primary alcohols therefore fell into two groups: those of chain length C_{10} – C_{12} supporting good growth, divided at C_9 (no growth in 24 h) from the shorter compounds giving moderate growth. A generally similar grouping was observed after 24 h growth on racemic secondary alkan-2-ols (Fig. 1b), with the exceptions that (i) the optimum chain-length was one carbon higher (C_{12} – C_{13}) than for primary alcohols, (ii) the critical low-growth chain-length was also one carbon higher (C_{10}) and (iii) all shorter alkan-2-ols gave very feeble growth. The reason why

Fig. 1. Extent of growth of *Pseudomonas* C12B on various primary and secondary alcohols. Culture optical densities (420 nm) were measured after growth on (a) homologous primary alcohols; (b) homologous alkan-2-ols; (c) isomeric heptanols (C₇), octanols (C₈), nonanols (C₉), decanols (C₁₀) and undecanols (C₁₁). In (c), the abscissa notation indicates the position of the OH group on the alkyl chain. Racemic mixtures of asymmetric secondary alcohols were used in all experiments except where indicated by the D and L notation. ■, Growth after 24 h; □, further growth in the 24–48 h period. ND, Not determined. In the absence of added alcohol, culture optical densities remained below 0.01.

nonan-1-ol and decan-2-ol should yield such low growth rates compared with close homologues is not clear. However, the one carbon shift between patterns for primary and alkan-2-ols is very reminiscent of the shift in free energy of binding of primary and alkan-2-ols to alcohol dehydrogenases, and of the corresponding sulphate esters to alkylsulphatases (see Dodgson & White, 1983, for a review). Such shifts have been interpreted as reflecting hydrophobic binding of only the longer alkyl segment attached to the hydroxylated or esterified carbon atom, so that, for example, binding of octan-2-ol ($C_6H_{13} \cdot CHOH \cdot CH_3$) is equal to that of heptan-1-ol ($C_6H_{13} \cdot CHOH \cdot H$), both binding via a C_6 alkyl segment. The growth patterns for homologous primary and alkan-2-ols may be reflecting a similar hydrophobic contribution at one or more stages in alcohol uptake and/or metabolism.

Fig. 1c shows the results of separate experiments in which growth after 24 and 48 h was measured for all primary and secondary isomers in the C_7 – C_{10} range, and also for some undecanols. Growth after 24 h on primary alcohols agreed with the pattern in Fig. 1a with excellent growth on C_{10} and C_{11} , modest growth on C_7 and C_8 and very little growth on C_9 alcohols. Extending the incubation period to 48 h allowed further growth to occur; this was most noticeable for nonan-1-ol which yielded growth commensurate with its C_8 and C_7 homologues.

Similarly consistent results were obtained for alkan-2-ols, and again, in the extended 48 h growth period, decan-2-ol supported growth comparable with that observed for its lower homologues. Interestingly, in the case of octan-2-ol, *Pseudomonas* C12B grew on the D-isomer, albeit poorly, but not at all on the L-isomer. Unfortunately, resolved enantiomers of other secondary alcohols were not available. Growth also occurred with some higher secondary alcohols, and was most prolific with secondary undecanols (Fig. 1c).

While further studies are required to establish growth kinetic parameters (lag-period, doubling times, maximum growth) as a function of growth-supporting alcohol, two important points have emerged from these experiments: the versatility of *Pseudomonas* C12B in its capacity to grow on a wide range of alcohols, typical of those produced by the organism's alkylsulphatases when confronted with a mixture of alkyl sulphate surfactants, and the ability of *Pseudomonas* C12B to grow on certain symmetrical or near-symmetrical alcohols, implying the operation of an alcohol dehydrogenase that could act on the products of the S3 alkylsulphatase.

Alcohol dehydrogenase activities

Extracts of cells grown to late-exponential phase on a variety of carbon sources were assayed (using NAD^+ as co-factor) for ability to oxidize octan-1-ol, D- and L-octan-2-ol, DL-octan-3-ol, DL-octan-4-ol and the symmetrical nonan-5-ol. Growth on nutrient broth (Fig. 2a), pyruvate (Fig. 2b), or the primary alcohol undecan-1-ol (Fig. 2c) led to production of appreciable dehydrogenase activity towards octan-1-ol and D-octan-2-ol but not towards the other secondary alcohols tested. An almost identical pattern was evident for extracts of cells grown on other substrates based on a primary alkyl chain, namely undecanoate or SDS (not shown). The same activities were present following growth on racemic secondary alcohols (the 2-, 3- and 6-undecanols, Fig. 2d–f) but a new feature now emerged: additional dehydrogenase activity was also detected towards the more symmetrical alcohols DL-octan-3-ol, DL-octan-4-ol and especially nonan-5-ol. Movement of the OH group in the growth-supporting alcohol towards the centre of the alkyl chain (undecanols substituted at $C-2 > C-3 > C-6$) had the effect of increasing the specific activity of extracts towards the symmetrical nonan-5-ol. Growth on undecan-6-ol and undecan-6-one produced closely similar patterns (Fig. 2f, g). It thus appeared that a new enzyme(s) with activity towards symmetrical alcohols was inducible by growth on secondary alcohols (and probably ketones), especially the more symmetrical isomers.

Chain length specificities of alcohol dehydrogenase activities

Pseudomonas C12B was grown to late-exponential phase in separate cultures using as carbon source hexan-1-ol, undecan-1-ol, undecan-6-ol or tridecan-2-ol. These alcohols were chosen as representatives of different types and for their ability to support good growth (Fig. 1). Cell extracts were prepared and assayed for dehydrogenase activity towards homologous series of primary alcohols, alkan-2-ols and symmetrical alcohols.

Fig. 2. Primary and secondary alcohol dehydrogenase activities in extracts of *Pseudomonas* C12B after growth on (a) nutrient broth; (b) pyruvate; (c) undecan-1-ol; (d) undecan-2-ol; (e) undecan-3-ol; (f) undecan-6-ol; (g) undecan-6-one. Separate assays were done with C_8 and C_9 alcohols; the abscissa notation indicates the positional isomers used. Racemic mixtures of asymmetric secondary alcohols were used except where indicated by the D and L notation.

Extracts assayed for activity towards a range of primary alcohols (C_2 – C_{11}) all produced the same profile irrespective of growth-supporting alcohol, with two peaks of activity separated by a trough at C_4 (Fig. 3*a–d*). The consistency of the enzyme activity–chain length profile with different alcohol growth-substrates was further evidence for constitutive synthesis of the primary alcohol dehydrogenase(s).

When the same extracts were assayed for activity towards alkan-2-ols, a quite different picture emerged (Fig. 3*e–h*). Growth on primary alcohols (C_6 and C_{11} , Fig. 3*e, f*) produced some activity towards C_3 and C_4 alkan-2-ols but this decreased as the chain-length was increased. Growth on secondary alcohols (undecan-6-ol or tridecan-2-ol, Fig. 3*g, h*), however, yielded good activities in the C_3 – C_{11} range, peaking in the C_7 – C_8 region. The results clearly indicated induction of one or more secondary alcohol dehydrogenases when the organism was grown on secondary alcohols.

Dehydrogenase activity towards symmetrical alcohols was highest in extracts grown on the symmetrical ketone undecan-6-one (Fig. 2*g*). When fresh extracts of cells grown on undecan-6-one were assayed for ability to oxidize homologous symmetrical alcohols, there was low activity towards heptan-4-ol, but more activity towards the C_5 , C_9 and C_{11} homologues (Fig. 4*a*). There was also remarkably high activity towards propan-2-ol, possibly because this alcohol is symmetrical and an alkan-2-ol and therefore a substrate for both groups of secondary alcohol

Fig. 3. Alcohol chain length specificity of primary alcohol dehydrogenase and alkan-2-ol dehydrogenase activities in extracts of *Pseudomonas* C12B grown on (a, e) hexan-1-ol; (b, f) undecan-1-ol; (c, g) undecan-6-ol; (d, h) tridecan-2-ol. (a)–(d), Dehydrogenase activities towards homologous primary alcohols; (e)–(h), dehydrogenase activities towards homologous alkan-2-ols.

Fig. 4. Alcohol chain length specificity of (a) symmetrical, (b) primary and (c) alkan-2-ol alcohol dehydrogenase activities in extracts of *Pseudomonas* C12B grown on undecan-6-one.

dehydrogenases (Fig. 4*a*, *c*). High activity towards propan-2-ol was accompanied by high activity towards its C₄ and C₅ homologues (Fig. 4*c*), and in this respect, the profile differs from that of cells grown on undecan-6-ol (Fig. 3*g*) and tridecan-2-ol (Fig. 3*h*). At longer chain lengths (> C₆), however, a consistent pattern peaking around C₇–C₈ was observed for cells grown on undecan-6-one (Fig. 4*c*), undecan-6-ol (Fig. 3*g*) or tridecan-2-ol (Fig. 3*h*). Growth on undecan-6-one also led to the usual pattern for primary alcohol dehydrogenase (Fig. 4*b*, cf. Fig. 3*a–d*).

Clearly (and not unexpectedly) *Pseudomonas* C12B produces a complex pattern of alcohol dehydrogenase activities, and several important features deserve comment. First, there was constitutive production of primary alcohol dehydrogenase activity that enabled the organism to oxidize alcohols liberated from primary alkyl sulphates by the P1 and P2 alkylsulphatases. Several other organisms are known to produce similar long-chain alcohol dehydrogenases (Dodgson & White, 1983). Second, constitutive production of D-alkan-2-ol dehydrogenase enables the organism to oxidize the products of the S2 (L-specific) alkylsulphatase. The D-alkan-2-ol dehydrogenase and the constitutive primary enzyme may be a single protein, as appeared to be the case in *C. terrigena*. Third, growth on secondary alcohols induces synthesis of an L-alkan-2-ol dehydrogenase. Dehydrogenase specificity for simple L-alkan-2-ols was first discovered in *C. terrigena* (Barrett *et al.*, 1980*b*) and has since been found by Hou *et al.* (1981*a*, *b*). In *Pseudomonas* C12B, the activity provides a route for oxidation of the bond-inverted products of the S1 (D-specific) alkylsulphatase. These sulphatase–dehydrogenase pairings have parallels in *C. terrigena* (see Introduction). Fourth, an enzyme with high activity towards symmetrical alcohols was inducible by growth on secondary (especially symmetrical) alcohols. The appearance of the symmetrical alcohol dehydrogenase always coincided with induction of the L-alcohol dehydrogenase and these may in fact be activities of a single broad-specificity enzyme. The corresponding activities in *C. terrigena* also appeared to be coincident, although the symmetrical activity was relatively weak. Symmetrical alcohol dehydrogenase activity has been reported in another *Pseudomonas* sp., but this lacked activity towards alkan-2-ols (Niehaus *et al.*, 1978). Discovery of symmetrical alcohol dehydrogenase activity in *Pseudomonas* C12B should be viewed in the light of earlier work from these laboratories on the inducible S3 alkylsulphatase that acts on symmetrical alkyl sulphates to yield the corresponding secondary alcohols. Induction of the S3 enzyme is intriguing because it requires a combination of a long-chain secondary alkyl sulphate and a secondary alcohol (Dodgson *et al.*, 1974). The observation that growth on secondary alcohols served also to induce the dehydrogenase required to oxidize the symmetrical alcohol products of S3 alkylsulphatase action suggested that synthesis of the enzymes might be co-ordinated. Work to explore the genetic relationships among the various alkylsulphatases and alcohol dehydrogenases has been initiated.

Finally, the observation that extracts of organisms grown on DL-undecan-2-ol (Fig. 2*d*) had dehydrogenase activity towards D- but not L-octan-2-ol was consistent with the capacity of the organism to grow on D-octan-2-ol but not on the corresponding L-isomer (Fig. 1*c*). Moreover these observations collectively suggested that growth on racemic DL-alkan-2-ols should result in assimilation of the D-isomer only, and simultaneous accumulation of the L-isomer in the culture medium. The system would thus afford a convenient method of resolving L-isomers of racemic alkan-2-ols (an otherwise difficult chemical resolution) and this prospect is currently being pursued.

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