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Dissimilatory Nitrate Reduction by *Propionibacterium acnes*

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Propionibacterium acnes **P13 was isolated from human feces. The bacterium produced a particulate nitrate reductase and a soluble nitrite reductase when grown with nitrate or nitrite. Reduced viologen dyes were the preferred electron donors for both enzymes. Nitrous oxide reductase was never detected. Specific growth rates were increased by nitrate during growth in batch culture. Culture pH strongly influenced the products of dissimilatory nitrate reduction. Nitrate was principally converted to nitrite at alkaline pH, whereas nitrous oxide was the major product of nitrate reduction when the bacteria were grown at pH 6.0. Growth yields were increased by nitrate in electron acceptor-limited chemostats, where nitrate was reduced to nitrite, showing that dissimilatory nitrate reduction was an energetically favorable process in *P. acnes*. Nitrate had little effect on the amounts of fermentation products formed, but molar ratios of acetate to propionate were higher in the nitrate chemostats. Low concentrations of nitrite (ca. 0.2 mM) inhibited growth of *P. acnes* in batch culture. The nitrite was slowly reduced to nitrous oxide, enabling growth to occur, suggesting that denitrification functions as a detoxification mechanism.**

Nitrate is reduced under anaerobic conditions by a taxonomically diverse range of facultative and strictly anaerobic bacteria (17, 39). The two major routes of dissimilatory nitrate metabolism involve (i) reduction of nitrate to nitrite or ammonia and (ii) reduction of nitrate to gaseous products (nitrous oxide, dinitrogen gas), which is called denitrification (17, 38). Dissimilatory reduction of nitrate to nitrite (nitrate respiration) is important to a number of bacteria, since the process involves energy conservation through generation of a proton motive force (14, 15) or by increased substrate-level phosphorylation reactions (16, 24).

Large amounts of nitrate are consumed in Western diets (25). However, data on the amount of nitrate entering the human colonic ecosystem are equivocal (4, 40, 41). There is increasing evidence for generation of nitrate by mammalian tissues (19, 30). Nitrate is rapidly metabolized by mixed populations of intestinal bacteria (1), and it is possible that the colon functions as a sink for this metabolite (43).

Many species of propionibacteria reduce nitrate (9, 12, 18). These bacteria are found in a variety of habitats. In humans, cutaneous propionibacteria such as *Propionibacterium acnes* occur on the skin and in the mouth, genital tract, and large intestine (9). Numerically, propionibacteria are probably the most important nitrate-reducing species in the colon, where cell population densities are frequently in excess of 10^9 bacteria per g (dry weight) of contents (13, 33). Little is known of their ecology in the large gut, but it is thought that they may play a role in the metabolism of fermentation intermediates such as lactate (46).

Although dissimilatory nitrate metabolism has been investigated in the classical propionibacteria (20, 44, 45), few data are available on cutaneous propionibacteria similar to those occurring in the large bowel. In this paper we report studies in nitrate reduction by *P. acnes* P13, an obligately anaerobic species isolated from human feces.

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MATERIALS AND METHODS

Bacterium. *P. acnes* P12 was isolated from human feces as described by Macfarlane et al. (33). The identity of the bacterium was established on the basis of Gram stain, morphology, and fermentation products formed in peptone-yeast extract-glucose broth, as described by Holdeman et al. (18), and biochemical tests (27).

Enzyme measurements. Batch cultures of *P. acnes* were grown anaerobically in serum bottles (280-ml capacity) in medium 1 (Wilkins-Chalgren broth [Oxoid Ltd.] plus 1 ml of Tween 80 per liter) in the presence and absence of KNO_3 (5 mM). The bacteria were harvested by centrifugation (20,000 × *g*, 30 min) and disrupted by two passages through a French pressure cell (1.1×10^5 kPa). Soluble and particulate fractions from the cell extracts were prepared as described by Dunn et al. (10). Nitrate reductase (NaR) activity was determined by the method of Lowe and Evans (31), with either methyl or benzyl viologen (50 mM) (both reduced with dithionite), formate (50 mM), fumarate (50 mM), malate (50 mM), lactate (50 mM), or succinate (50 mM) as the electron donor. Nitrate-dependent oxidation of NADH was measured as described by Macfarlane and Herbert (35). Nitrite reductase (NiR) was measured under anaerobic conditions (argon atmosphere) in Thunberg cuvettes by the method of Cole et al. (7). Nitrous oxide reductase was measured as described by Kristjansson and Hollocher (28).

Studies on nitrate reduction at different values of culture pH. *P. acnes* was grown on medium 1 as described above. Exponentially growing cultures were aseptically harvested by centrifugation (20,000 × *g*, 30 min). The cell pellets were suspended in medium 1, and samples (10 ml) of the suspensions were used to inoculate duplicate serum bottles (280-ml volume) containing medium 1 (230 ml) with and without KNO_3 (3 mM). To determine the effect of nitrite on bacterial growth, some bottles contained KNO_2 (0.2 mM) instead of nitrate. The media were buffered to pH 6.0, 6.5, 7.0, and 7.5 with 0.1 M sodium phosphate buffer. The bottles were immediately sparged with high-purity argon gas for 5 min via a three-way valve system before being incubated (37°C) on a rotary shaker. Acetylene (5%, vol/vol) was added to some bottles to determine whether nitrous oxide was further

reduced to nitrogen gas (2, 47). Samples of headspace gas (5 ml) and liquid samples (3 ml) were aseptically removed at 0, 1.5, 3, 6, 12, 24, 48, and 60 h by using gas-tight 10- and 5-ml plastic syringes, respectively. The pH of liquid samples measured at the beginning and end of all incubations showed that no significant change in pH had occurred. The samples were replaced by adding 8 ml of argon through a 0.2-μm-pore size filter. Initial experiments showed that gas could be stored in the syringes for up to 6 h without significant loss.

Growth in continuous culture. Bacteria were grown in 0.5-liter (working volume) glass chemostats at 37°C. Anaerobic conditions were maintained by sparging the cultures with an H_2-CO_2-N_2 gas mixture (10:10:80). Temperature and pH (6.5) were controlled as described by Macfarlane and Englyst (34). The medium (medium 2) contained the following (grams per liter): peptone water, 5.0; tryptone soya broth, 5.0; cysteine, 0.5; $NaHCO_3$, 5.0; sodium lactate, 2.3; Tween 80, 1.0; medium 2 also contained the following (milligrams per liter): $FeSO_4 \cdot 7H_2O$, 0.1; hemin, 1.0; adenine, 1.0; guanine, 20.0. KNO_3 was added to some bottles to give a final concentration of 0.3 g liter^{-1}. The filter-sterilized salts solution (10 ml liter^{-1}) (11) and vitamin solution (10 ml liter^{-1}) of Ferguson and Cummins (12) were added after autoclaving.

Chemical analyses. Bacteria were removed from samples by centrifugation (27,000 × *g*, 10 min). Nitrite was determined by the method of Barnes and Folkard (3). Nitrate was enzymatically reduced to nitrite by using a membrane preparation containing NaR from *Escherichia coli*. Nitrate concentrations were determined by difference with the nitrite measurements. Volatile fatty acids and other carboxylic acids were detected by gas chromatography as described by Holdeman et al. (18).

Gas analyses. Nitrous oxide was measured by gas chromatography with a Pye Series 104 gas chromatograph equipped with a 1-ml injection loop. The gas was separated on a 3-foot (ca. 90-cm) glass column (4-mm inner diameter) containing Porapack Q (Waters Associates). Argon was the carrier gas, and detection was by a thermal conductivity detector connected to a Pye Unicam CDP1 computing integrator. The peaks were quantified by comparison with authentic gas standards.

Dry weight measurements. Dry weights were measured as described by Keith and Herbert (23).

Protein estimations. The method of Lowry et al. (32) was used, with bovine serum albumin as the standard.

Chemicals. All chemicals were obtained from Sigma Chemical Co., with the exception of the formulated bacteriological media (Oxoid Ltd.).

RESULTS

Nitrate and nitrite reductases. *P. acnes* produced a particulate NaR and a soluble NiR during growth with nitrate. Methyl viologen, benzyl viologen, lactate, and NADH were good electron donors for NaR (82.0 ± 9.6, 99.7 ± 3.7, 23.2 ± 2.8, and 17.3 ± 4.2 nmol of nitrite produced min^{-1} mg of protein^{-1}, respectively; means of three determinations ± standard errors of the means), but low activities (<5 nmol of nitrite produced h^{-1} mg of protein^{-1}) were recorded with malate, fumarate, succinate, and formate. Reduced viologen dyes were the sole electron donors for NiR (for benzyl and methyl viologen, 9.8 ± 1.9 and 22.4 ± 8.8 nmol of nitrite produced min^{-1} mg of protein^{-1}, respectively). In all subsequent experiments, benzyl viologen was used as the electron donor in NaR assays and methyl viologen was used for assays of NiR. Attempts to measure nitrous oxide reductase by using a variety of electron donors were never successful, although high levels of nitrous oxide reductase were found in control assays with cell extracts from the denitrifying bacterium *Pseudomonas aeruginosa*.

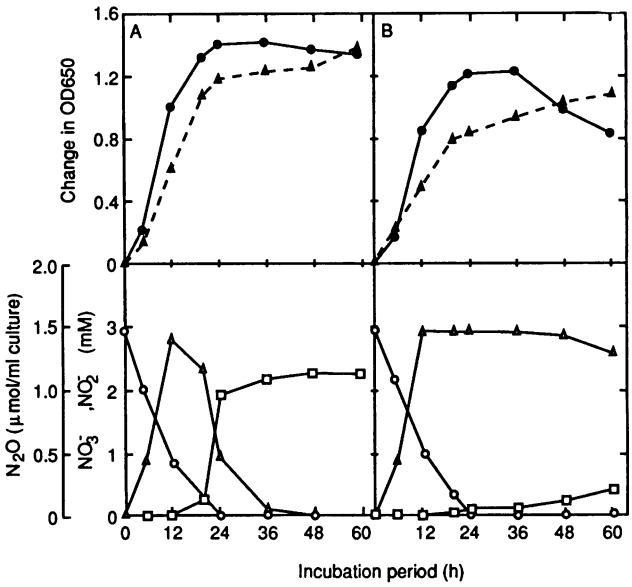

FIG. 1. Effect of pH on nitrate reduction by *P. acnes*. Bacteria were grown in batch culture on medium 1 at either pH 6.0 (A) or pH 7.5 (B) in the presence and absence of KNO_3 (3 mM). Results are the means of two separate experiments. Symbols: ▲, growth without nitrate; ●, growth with nitrate; ○, nitrate; △, nitrite; □, nitrous oxide.

Effect of culture pH on nitrate reduction. Specific growth rates of *P. acnes* were increased by nitrate from 0.33 h^{-1} to 0.46 h^{-1} during growth at pH 6.0 and from 0.18 h^{-1} to 0.38 h^{-1} in pH 7.5 cultures. Nitrate was completely metabolized irrespective of culture pH; however, pH influenced the products of nitrate reduction (Fig. 1). At pH 7.5, nitrate was stoichiometrically reduced to nitrite within 12 h. Nitrite was the principal product of nitrate reduction, but some nitrous oxide was formed toward the end of active growth. In contrast, nitrite accumulation was transient during growth at pH 6.0 and nitrous oxide was the major product of nitrate metabolism. Acetylene blocking did not increase levels of nitrous oxide produced, indicating that nitrate was not denitrified beyond nitrous oxide (data not shown). The influence of pH on denitrification was confirmed in subsequent experiments, which demonstrated that nitrous oxide formation decreased concomitantly with increasing culture pH (Fig. 2).

Nitrate reduction by continuous cultures. Measurements of enzyme activities in lactate-limited chemostat cultures showed that NaR was not significantly affected by bacterial growth rate, but that NiR activities decreased as culture dilution rates were increased (Table 1). Nitrate was almost completely converted to nitrite, showing that denitrification was a minor process and indicating that the cultures were limited with respect to the inorganic electron acceptor. Measurements of fermentation products showed that pyruvate accumulated in nitrate cultures and that concentrations were greatest at high dilution rates. Lactate was never found in culture spent media. Except at a dilution rate of 0.1 h^{-1}, at which the nonnitrate cultures were washing out, nitrate had little effect on the total amounts of fermentation prod-

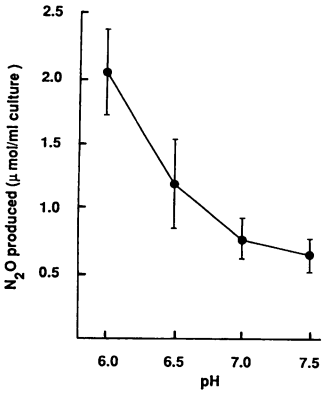

FIG. 2. Effect of pH on nitrous oxide production by *P. acnes*. The bacterium was grown in batch culture on medium 1 with KNO_3 (3 mM) for 48 h. Data are the means of three experiments ± standard errors of the means.

ucts formed. However, cell yields and acetate/propionate molar ratios were higher in the nitrate chemostats at all dilution rates.

Influence of nitrite on growth of *P. acnes* in batch culture. Low concentrations of nitrite (0.2 mM) markedly inhibited growth of *P. acnes* (Fig. 3). Significant growth did not occur until about 24 h after inoculation in the nitrite cultures, and this was accompanied by rapid reduction of the nitrite to nitrous oxide.

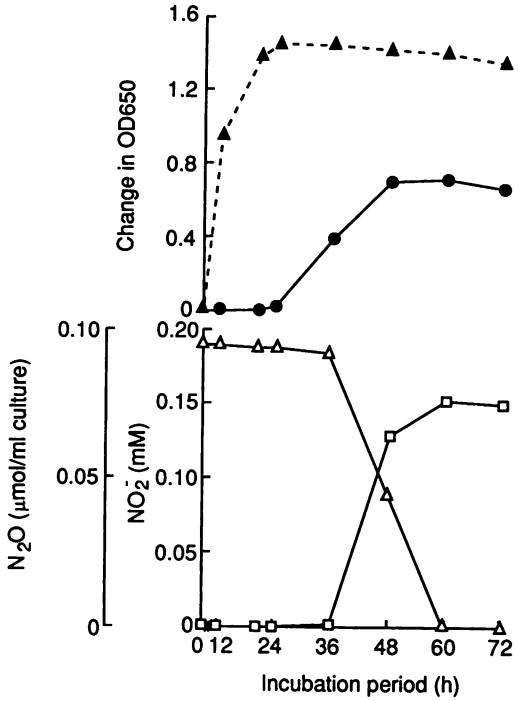

FIG. 3. Influence of nitrite on growth of *P. acnes* in batch culture. Bacteria were grown at pH 6.0 in medium 1 in the presence and absence of nitrite (0.2 mM). Results are representative of two separate experiments. Symbols: ▲, growth without nitrite; ●, growth with nitrite; △, nitrite; □, nitrous oxide.

DISCUSSION

Most dissimilatory nitrate reductases are inducible enzymes (38, 39); however, in many propionibacteria NaR, although stimulated by nitrate, appears to be constitutive (20, 44). The particulate NaR of *P. acnes* P13 differs from the enzyme in other propionibacteria in that nitrate is required for its induction (Table 1). A variety of electron donors were used by *P. acnes* NaR, but the most efficient were benzyl viologen, methyl viologen, lactate, and NADH. These electron donors were also found to be active with the particulate NaR of *Propionibacterium acidi-propionici* (21).

Nitrate was rapidly reduced to nitrite by batch cultures of *P. acnes*. Nitrite accumulated initially but was eventually reduced at a much slower rate to nitrous oxide by a soluble NiR. Nitrous oxide was the end product of nitrate metabolism by the bacterium during growth at pH 6.0, whereas nitrite was the principal product at pH 7.5. Studies have shown that in many natural environments denitrification is favored by conditions of alkaline pH (26). However, in pure cultures of *Paracoccus denitrificans* NiR activity and nitrous oxide accumulation are greatest when the cells are incubated under acidic conditions (29). The influence of pH on nitrate reduction by *P. acnes* may be important in the large bowel, where the pH of gut contents is commonly in the range of 5.5

TABLE 1. Effect of nitrate on metabolism of *P. acnes* grown in continuous culture with lactate (20 mM) as the carbon source[a]

Dilution rate (h^{-1})	Nitrate (5 mM) in growth medium	Enzyme activity		Concn (mM)		Fermentation products (mM)					Culture dry weight (g $liter^{-1}$)
		NaR[b]	NiR[c]	Nitrate	Nitrite	Acetate	Propionate	Isovalerate	Pyruvate	Total	
0.02	−	ND[d]	ND	ND	ND	4.3	6.2	3.0	ND	13.5	0.18
	+	18.5	6.7	ND	4.6	4.7	5.2	2.8	0.8	13.5	0.23
0.05	−	ND	ND	ND	ND	4.7	8.2	1.9	ND	14.8	0.29
	+	22.0	4.3	ND	4.8	5.7	6.1	2.4	0.5	14.7	0.34
0.08	−	ND	ND	ND	ND	3.6	8.8	1.6	ND	14.0	0.60
	+	18.0	3.9	ND	5.0	6.8	6.5	0.9	0.9	15.1	0.68
0.10	−	ND	ND	ND	ND	0.9	2.1	ND	ND	3.0	0.09
	+	16.0	1.2	ND	4.9	4.4	4.4	0.5	2.1	11.4	0.75

[a] Results are means from two separate experiments.
[b] Nanomoles of nitrite produced per minute per milligram of protein.
[c] Nanomoles of nitrite reduced per minute per milligram of protein.
[d] ND, Not detected (less than 0.1 mM).

to 6.0 in the right colon but neutral to alkaline in the left colon (8).

Specific growth rates of *P. acnes* were more than 30% higher during growth with nitrate in batch cultures. This contrasts with observations made by Kaneko and Ishimoto (20), who found that nitrate had little effect on growth rates of *P. acidi-propionici*. However van Gent-Ruijters et al. (45) considered that the stimulation of growth rates by nitrate found with "*Propionibacterium pentosaceum*" (now classified as *P. acidi-propionici* [9]) was due to increased lactate metabolism in nitrate cultures.

Growth inhibition by low concentrations of nitrite in batch culture (Fig. 3) suggests that nitrite reduction may be a detoxification mechanism in *P. acnes*, in common with the classical propionibacteria studied by Kaspar (22). This conclusion is supported by rates of nitrous oxide production in batch cultures, which range from 4 to 32 nmol h^{-1} mg of $bacteria^{-1}$. These denitrification rates are considerably lower than activities found in other denitrifying species, as typified by *P. denitrificans* (ca. 9 μmol of nitrogen produced h^{-1} mg of $bacteria^{-1}$) (37). When *P. acnes* was grown with 5 mM nitrate in continuous cultures, the nitrate was almost completely converted to nitrite. These data showed that denitrification was insignificant, and this correlated with the low levels of NiR activity (Table 2). Unlike the batch cultures, in which nitrite concentrations as low as 0.2 mM inhibited growth, the bacteria were not inhibited by the considerably higher amounts of nitrite present in the chemostats. This could be explained by selection of nitrite-resistant populations or, alternatively, by the ability of faster-growing propionibacteria to tolerate nitrite as suggested by Kaspar (22).

The major fermentation products of propionibacteria are acetate, propionate, and carbon dioxide. Acetate and carbon dioxide are formed by oxidative decarboxylation of pyruvate, whereas propionate is produced from pyruvate by the succinate pathway (Fig. 4). Nitrate strongly influenced fermentation product formation in continuous cultures. Acetate/propionate molar ratios increased during growth with nitrate, and pyruvate accumulated, particularly at high growth rates (Table 1).

Reduced pyridine nucleotides and flavoproteins are required to convert pyruvate to succinate (Fig. 4). The reduction of propionate formation indicates that when nitrate functions as a terminal electron acceptor, NADH and reduced flavoprotein are preferentially used to reduce nitrate to nitrite, with the result that propionate production is repressed. This has previously been reported to occur in "*P. pentosaceum*" (44). That study also showed that acetate formation from lactate was not affected during growth with nitrate. However, nitrate increased acetate production by *P. acnes*, showing that there was stimulation of the phosphoroclastic system. This was also found by Kameko and Ishimoto in studies with *P. acidi-propionici* (20).

Cell yields of *P. acnes* were significantly higher (12 to 22%) in the nitrate chemostats, demonstrating that the metabolism of nitrate was an energetically favorable process. Dissimilatory reduction of nitrate to nitrite is energetically advantageous for many species of facultative and strictly anaerobic bacteria. In klebsiellas, vibrios, and enterobacteria, energy is generated by respiratory processes during nitrate reduction (5–7, 10, 35); whereas with the clostridia, nitrate is reduced to nitrite by a soluble NaR, and extra ATP is generated by substrate level reactions (16, 24). In nitrate-grown "*P. pentosaceum*," energy transduction was by electron transport phosphorylation (45), and an

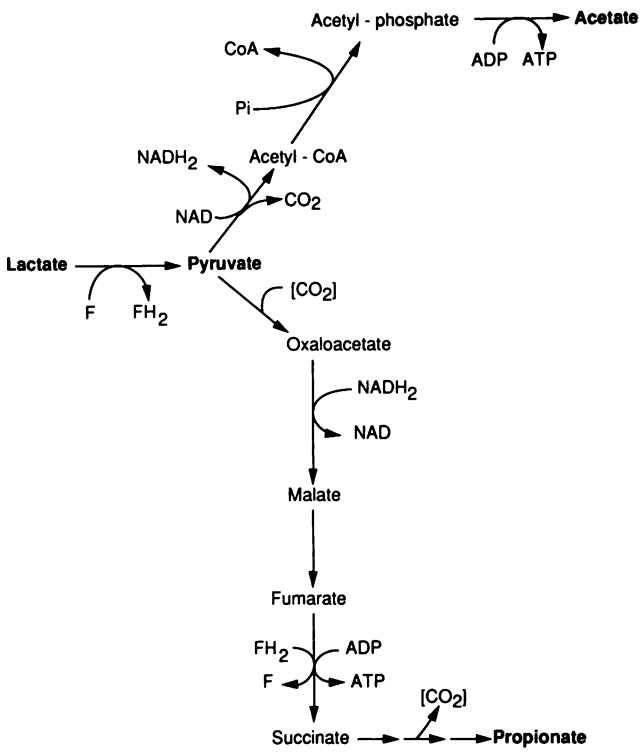

FIG. 4. Fermentation pathways of *P. acnes* growing on lactate.

electron transport system has been demonstrated in "*P. arabinosum*" (42). The enzymological studies, fermentation results, and cell yield data obtained here indicate that this form of energy transduction also occurs in *P. acnes*.

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