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Enantioselective catabolism of racemic serine: preparation of D-serine using whole cells of *Fusobacterium nucleatum*

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ABSTRACT

Incubation of racemic serine with the anaerobic bacterium *Fusobacterium nucleatum* yielded D-serine in high enantiomeric excess (>95%). Selective degradation of the L-amino acid was most efficient when *F. nucleatum* was resuspended in a buffer at high cell densities (ca. 50–100 g damp cells/L); a single incubation effectively removed almost all L-serine from racemic mixtures at initial concentrations up to 800 mM, the solubility limit. The product D-amino acid was separated from the metabolic end-products (acetate, butyrate and lactate) and buffer components by a single cation-exchange step. After recrystallization, 83% of the D-serine in the initial racemate was recovered with >99% ee (HPLC) and 98% purity (HPLC). This anaerobic microbial approach provides a viable complementary method for generating D-serine, a valuable chiral starting material for chemical synthesis.

1. Introduction

As primary members of the chiral pool,[1] α-amino acids are valuable substrates for the synthesis of chiral compounds.[2–4] Of the common amino acids, the individual enantiomers of serine are particularly versatile substrates for chemical synthesis,[4,5] serving as starting points for the preparation of important chiral synthons, such as Garner's aldehyde[6] and various other serine derivatives.[3,7]

While D-amino acids have been prepared by controlling the oxidation state and reactivity of the terminal carbons in the backbone of L-serine,[8] the more expensive D-enantiomer of serine also has been a preferred starting point for the synthesis of chiral compounds of diverse structures and properties. For example, over the past few years, D-serine has been used for the synthesis of enzyme inhibitors,[9] nonproteinogenic amino acids,[10] neuroexcitatory kainoid amino acids,[11] lysinoalanine,[12] the cyclopeptide alkaloid paliurine,[13] substituted nitrogen-containing heterocycles,[14] sphingosine,[15] and conformationally constrained analogs of diethylenetriaminepentaacetic (DTPA) acid used as ligands in radiopharmaceuticals and contrast agents for medical imaging.[16]

Although fermentation is a common source of L-amino acids,[17] only a few examples of the excretion and accumulation of D-amino acids in microbial cultures are known [e.g., D-alanine by *Corynebacterium fasciens*[18] and γ-poly(glutamic acid) enriched in D-glutamate by *Bacillus* strains[19]]. As an alternative, the enantioselective catabolism of L-amino acids has been utilized to isolate unmetabolized D-amino acids from microbial cultures supplemented with less expensive and readily available racemic amino acids. In this way, D-alanine (*Candida maltosa*[20] and *Arthrobacter* sp. NJ-26[21]), D-α-aminoadipic acid (*Pseudomonas putida*),[22] D-aspartic acid (*Pseudomonas dacunhae*),[23] (*R*)-baclofen (*Streptomyces halstedii*),[24] branched chain D-amino acids (*Proteus vulgaris*),[25] D-glutamic acid (*Lactobacillus brevis*),[26] D-histidine (*Proteus vulgaris*),[25] D-homoserine (*Arthrobacter nicotinovorans*),[27] D-lysine (*Comamonas testosteroni*),[28] D-methionine (*Proteus vulgaris*),[29] D-ornithine (*Proteus vulgaris*),[25] D-proline (*Candida* sp. PRD-234)[21] and the aromatic amino acids D-phenylalanine (and ring-substituted analogs), D-tyrosine and D-tryptophan (*Rhodotorula graminis* homogenate)[30] have been obtained in high yield and enantiomeric excess.

In other instances, genetic modifications have incorporated the gene for D-amino acid transaminase into *Escherichia coli* for the production of D-phenylalanine and other D-amino acids,[31] and enhanced glutamate racemase (*E. coli*)[21,32,33] and L-serine deaminase (*E. coli*)[34] activities for the preparation of D-glutamate, D-phenylalanine and D-serine. Most recently, the incorporation of a gene encoding an amino acid racemase into L-amino acid producing strains of *Corynebacterium glutamicum*[35] resulted in the extracellular accumulation of similar amounts of the D- and L-enantiomers of arginine, lysine and ornithine. The modified L-serine producing strain, however, excreted more D-serine (81 mM; vs 37 mM L-serine), and the enantiomeric enrichment was attributed to the specific transport of D-serine.

An alternative microbial approach is indicated by the preferred utilization of amino acid substrates by several anaerobic

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Fusobacterium species.[36–39] The rapid, efficient and selective utilization of L-serine[36] demonstrated the ability of *Fusobacterium nucleatum* to remove one isomer when supplied with a racemic amino acid. Herein, we demonstrate the gram-scale feasibility of using this readily available, stable anaerobic bacterium to prepare D-serine from racemic serine, a strategy first realized when Pasteur obtained 'tartrique gauche' from racemic tartaric acid using yeast.[40]

2. Results and discussion

2.1. Development of bacterial resuspension conditions

During growth on peptone medium, *F. nucleatum* preferentially and efficiently utilized the L-enantiomer of serine.[36] The peptone medium, however, is a complex mixture, and a large proportion of the medium constituents remain after the growth of *F. nucleatum* is complete. Rather than attempting to isolate D-serine from this complicated matrix, *F. nucleatum* cells grown on peptone medium were collected and resuspended[20,26] in a buffered aqueous solution of salts, vitamins and racemic amino acid at pH 7.4,[41] where they remained viable under anaerobic conditions for at least three days.

By using an anaerobic organism, an exchange of atmospheric gases with individual cells is neither needed nor desired, and the cell density attained by growth of *F. nucleatum* on peptone medium (yield of approximately 5 g damp cells/L) could be concentrated 5- to 40-fold by varying the volume of the resuspension buffer. At approximately 200 g damp cells/L, however, the resuspension mixture became very viscous and difficult to manipulate. Typical experiments were conducted at 50 g damp cells/L, and the rate of L-serine utilization was higher than that observed during the growth of *F. nucleatum* on the peptone medium.[36] In order to optimize the incubation parameters, samples from resuspension mixtures were removed at time intervals and centrifuged; the supernatants were analyzed by HPLC to determine the utilization of serine and the enantiomeric excess (ee) of the residual D-amino acid.

In a static resuspension experiment (330 mM DL-serine; 35 g damp cells/L), D-serine in the culture fluid reached 59% and 85% ee, after 16 and 24 h, respectively. By contrast, D-serine at 99% ee was present at both 16 and 24 h in a parallel, magnetically stirred resuspension mixture. The agitation may have overcome the tendency of *F. nucleatum* to aggregate,[42] promoting faster and more complete utilization of L-serine. All subsequent resuspension experiments, therefore, were stirred.

In a series of incubations of DL-serine (initial concentrations of 20–900 mM) with resuspended *F. nucleatum* cells, the serine concentration decreased to approximately 50% of the initial value within 16 h (Table 1). At higher initial concentrations of DL-serine (i.e., 330, 500, 700, and 900 mM), D-serine in high enantiomeric excess (i.e., 99%, 95%, 86%, and 95% ee, respectively) remained, confirming the rapid and efficient utilization of L-serine and the ability of *F. nucleatum* to tolerate high concentrations of D-serine.[35] By contrast, incomplete utilization of the L-enantiomer was observed at similar concentrations of racemic methionine (1000 mM)[29] and mandelic acid (>300 mM),[43] and was attributed to product inhibition and enzyme inactivation. On the other hand, incomplete degradation of L-alanine by *Candida maltosa* was overcome by maintaining a constant pH for the resuspension buffer.[20]

In principle, the efficiency of D-serine preparation is greatest at high initial concentrations of the racemate. DL-Serine at 900 mM, however, crystallized from the autoclaved medium, defining a practical upper concentration limit for the initial racemic amino acid that is greater than the solubility of DL-serine in water (ca. 50 g/L or 475 mM).[44] Subsequent resuspension experiments were conducted at 800 mM DL-serine (i.e., 84 g/L). Similar initial concentrations of racemate have been employed in other whole-cell processes for the preparation of D-alanine (2250 mM[20] and 2400 mM[21]) D-α-aminoadipic acid (31 mM),[22] D-aspartic acid (2500 mM),[23] (*R*)-baclofen (14 mM),[24] branched chain D-amino acids (ca. 800 mM),[25] D-glutamic acid (680 mM),[26,32] D-histidine (650 mM),[25] D-homoserine (510 mM),[27] D-lysine (680 mM),[28] D-methionine (670 mM),[29] D-mandelic acid (300 mM),[43] D-ornithine (750 mM),[25] D-proline (870 mM),[21] and aromatic D-amino acids (ca. 6 mM).[30]

Table 1
Incubations of DL-serine with resuspended cells of *F. nucleatum*

Initial DL-serine (mM)	Cell density (g/L)	Initial DL-serine mmol per g damp cells	Time (h)	Residual serine % initial concn
20	15	1.3	12	52
60	22	2.7	12	39
100	26	3.8	12	61
200	26	7.7	12	57
330	35	9.3	16	54
500	50	10	14	57
700	50	14	14	62
900	50	18	14	65

At the times indicated, samples were removed and analyzed by HPLC to determine residual serine concentrations.

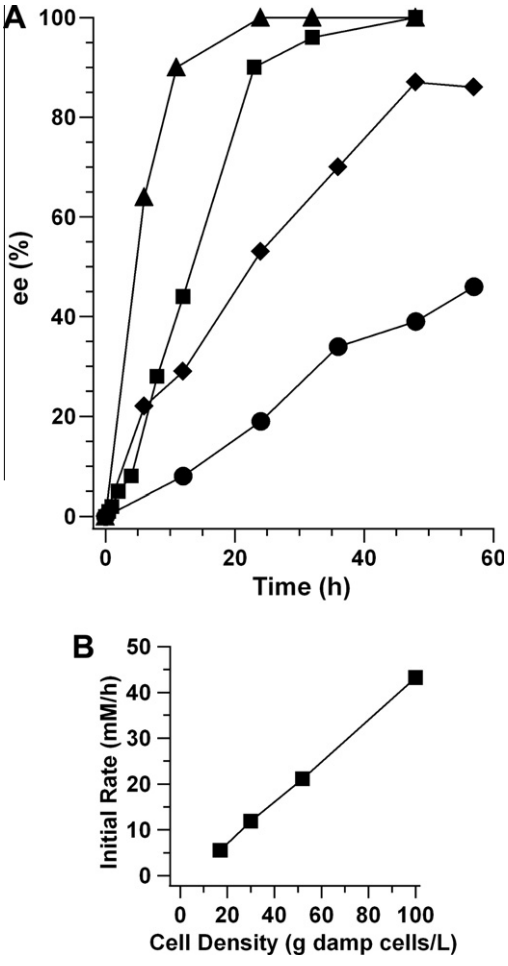


Figure 1. Effect of resuspended cell density on D-serine enrichment from an initial concentration of 800 mM DL-serine. (A) *F. nucleatum* was resuspended at 17 (●), 30 (♦), 52 (■), and 100 (▲) g damp cells/L. The corresponding loading factors were 47, 27, 15, and 8 mmol DL-serine/g damp cells. The ee (%) was determined by HPLC. (B) Variation of the initial rate of L-serine utilization with the density of resuspended cells.

The results of the initial experiments (Table 1) indicated that L-serine was utilized effectively when up to 18 mmol racemate per g damp *F. nucleatum* cells were provided. Whether larger loadings of racemic amino acid were possible was investigated systematically at 800 mM DL-serine. At cell densities from 17 to 100 g damp cells/L (Fig. 1A), the initial rate of L-serine utilization increased proportionally with increasing cell density (Fig. 1B), and similar specific rates of approximately 0.4 mM/h/g damp cells were observed. As L-serine utilization proceeded, only minor decreases in rate were observed (Fig. 1A), showing little or no inhibition by the accumulation of metabolic products (Section 2.2). Residual D-serine at >95% ee, however, was attained only at the two highest cell densities (Fig. 1A) corresponding to loading factors of 8 and 15 mmol DL-serine/g damp cells. At loading factors of 27 and 47 mmol DL-serine/g damp cells, a smaller proportion of L-serine was utilized, yielding D-serine with only 87% and 45% ee, respectively. Overall, the results indicate an upper limit of approximately 20 mmol DL-serine/g damp cells for the efficient utilization of L-serine, yielding D-serine at >95% ee.

The capacity of *F. nucleatum* cells to utilize larger amounts of L-serine was assessed by incubating *F. nucleatum* with 800 mM DL-serine and subsequently transferring the cells to fresh 800 mM DL-serine. In Expt. 1, residual D-serine of 97% ee was present at 35 h (Fig. 2), reproducing the previous results obtained under identical conditions (Fig. 1A). Upon resuspension of these 35-h cells in fresh 800 mM DL-serine, utilization of L-serine continued at about 40% of the initial rate determined in the first suspension (Fig. 2). Furthermore, the utilization of L-serine was incomplete, ultimately yielding a D-serine enriched mixture (47% ee). On the other hand, the continued utilization of L-serine in the second solution of racemate demonstrated that the capacity of the *F. nucleatum* cells was greater than the approximately 8 mmol L-serine/g damp cells utilized during the initial 35-h incubation. To make the most of the unused capacity of *F. nucleatum* cells for L-serine utilization and optimize the efficiency of D-serine production, a second resuspension might be more effective at a lower loading factor of racemic amino acid. Alternatively, the second resuspension might be effective in removing minor amounts of L-enantiomer from a partially resolved mixture.[45]

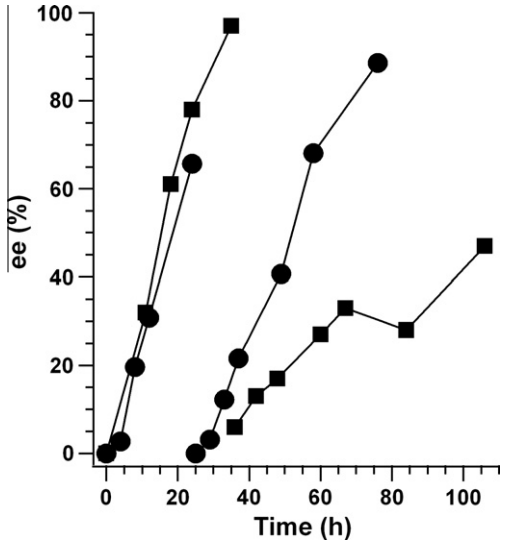


Figure 2. Progress of D-serine enrichment when *F. nucleatum* (52 g damp cells/L) was resuspended successively in 800 mM DL-serine. In Expt. 1 (■) and Expt. 2 (●), cells were transferred to fresh solutions of DL-serine at 24 and 35 h of incubation, respectively. The ee (%) was determined by HPLC.

In a duplicate experiment (Fig. 2, Expt. 2), the initial rate of L-serine utilization was similar to that in Expt. 1 (previous paragraph), further demonstrating the reproducibility of initial L-serine utilization under resuspension conditions. In Expt. 2, the cells were transferred to fresh 800 mM DL-serine at 24 h when approximately 80% of the initial L-serine had been utilized. After the transfer, the rate of L-serine utilization was about 70% of the initial rate, and residual D-serine of 89% ee was obtained. The total L-serine utilized in each experiment was similar, about 13 mmol L-serine/g damp cells. At the highest loading factors used in previous experiments (Fig. 1A), the total utilization of L-serine was 12 and 15 mmol/g damp cells. The similar values from the four independent experiments indicate an inherent capacity for L-serine utilization by *F. nucleatum* and define a maximum loading factor of approximately 26 mmol DL-serine/g damp cells. In the experiment conducted at 27 mmol DL-serine/g damp cells (Fig. 1A), the *F. nucleatum* cells functioned almost at maximum capacity, utilizing 12 mmol L-serine/g damp cells (i.e., 93% of the initial L-serine) and yielding D-serine in 87% ee.

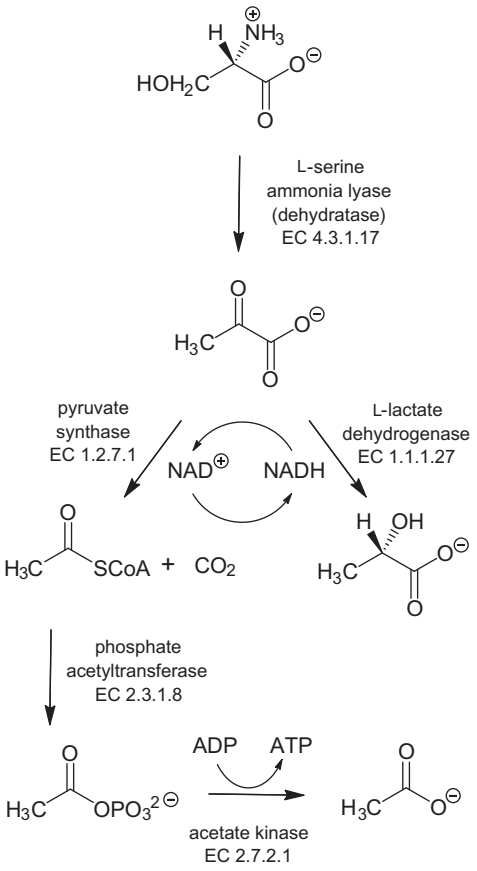
2.2. Identification of metabolic end-products

The supernatant from a resuspension experiment (initially 800 mM DL-serine) was titrated with aqueous NaOH to convert carboxylic acid end-products to nonvolatile salts and lyophilized.[46] In addition to residual serine, ^{1}H and ^{13}C NMR analysis of the lyophilized residue revealed acetate and lactate as major components, and a minor amount of butyrate (relative amounts approximately 10:10:1). The accumulation of these three carboxylic acids in cultures of *F. nucleatum* is well known, but various ratios have been reported,[37,39] perhaps reflecting different culture conditions. A few other resonances in the NMR spectra were just discernable above the baseline, validating the use of resuspension conditions to avoid complications during the isolation of D-serine after L-serine catabolism. The detection of carboxylic acids as the major metabolites suggested that simple cation-exchange chromatography would be sufficient to separate D-serine from metabolic products and residual media components.

2.3. Pathway of L-serine catabolism

The accumulation of lactate in cultures of *F. nucleatum* has been linked to serine catabolism,[47] and a pathway for the conversion of L-serine to the two major end-products, acetate and lactate, is presented in Scheme 1. In the *F. nucleatum* genome, the genes for pyruvate synthase, L-lactate dehydrogenase, phosphate acetyltransferase, and acetate kinase are clustered,[48] and the gene for L-serine ammonia-lyase (or dehydratase) has been annotated in the genome of three *F. nucleatum* subspecies.[48,49] The iron–sulfur enzyme L-serine ammonia-lyase has also been detected in a proteomic study of *Fusobacterium varium*[50] and purified from several species of anaerobic bacteria.[51] D-Serine is not a substrate for the isolated enzyme,[51] accounting for the enantioselectivity observed for serine catabolism by *F. nucleatum*. Given the documented instability of L-serine ammonia-lyase upon exposure to air,[51] the use of the isolated enzyme for L-serine degradation is less practical than the current whole cell approach using oxygen-tolerant whole cells of *F. nucleatum*.[52]

The observed accumulation of approximately equal quantities of acetate and lactate suggests that the oxidation of pyruvate to acetyl-CoA and carbon dioxide is balanced by the reduction of pyruvate to lactate (Scheme 1). Maintaining a balance between the oxidative and reductive processes provides a constant supply of NAD^+ as the electron acceptor for the oxidation of pyruvate to acetyl-CoA, enabling subsequent ATP generation. The small amount of butyrate formed suggests that the acetyl-CoA to butyrate reaction sequence[46,48] is less important for the replenishment of NAD^+ when pyruvate is readily available. In both the butyrate and lactate pathways, the coenzyme redox balance is maintained



Scheme 1. Catabolism of L-serine: regeneration of the coenzyme NAD^+ and the formation of acetate and lactate. Butyrate (not shown) is formed from acetyl-CoA.[46]

while one acetyl-CoA (i.e., one ATP) is generated from two molecules of pyruvate. Only a single enzyme-catalyzed step, however, is required for the reduction of pyruvate to lactate.

2.4. Preparation of D-serine

The viability of enantioselective catabolism for the preparation of D-serine was demonstrated under optimized conditions: 800 mM DL-serine; 5 g damp cells in 100 mL resuspension buffer; and an incubation time of 42 h. The supernatant from the incubation mixture was applied to the cation-exchange resin in order to separate the residual D-amino acid from the carboxylate anions. The treatment of amino acid containing fractions with charcoal and recrystallization from aqueous ethanol gave D-serine as an optically active white solid (83% recovery from the racemate; 98% pure by HPLC) with an ee greater than 99% (by HPLC).

3. Conclusion

Without genetic modifications, *F. nucleatum* demonstrated an inherent capacity to selectively catabolize large quantities of L-serine from a racemic mixture. Depletion of the L-amino acid was most efficient at high densities of resuspended bacterial cells, conditions easily achieved with the anaerobic bacterium. The acetate and lactate that accumulated in the culture fluids as major catabolic products were easily separated from residual D-serine, which was recovered in high yield and >99% ee. The optimized process has several advantages over a reductive animation process[53] employing isolated enzymes and the expensive, unstable β-hydroxypyruvate. Racemic serine is a low cost starting point; upon conversion of the racemate to D-serine, about a fivefold increase in economic value is realized. Overall, anaerobic biodegradation is an attractive alternative for the preparation of D-serine, a valuable member of the chiral pool.

4. Experimental

4.1. General

Melting points (uncorrected) were determined in open capillary tubes on a GallenKamp apparatus and optical rotations were measured on a Perkin-Elmer 141 polarimeter at 589 nm. The ^1H and ^{13}C NMR spectra (250.1 and 62.9 MHz, respectively) of samples dissolved in D_2O were acquired on a Bruker AC 250F spectrometer. Chemical shifts (δ) are reported in ppm referenced to HOD (4.80 ppm) for proton spectra and external dioxane (66.66 ppm) for carbon spectra. Multiplicities assigned to carbon resonances were determined from DEPT spectra. Electrospray ionization mass spectrometry (ESI-MS and ESI-MS/MS) was performed on a Thermo-Finnigan LCQ Duo ion trap using flow-injection (20 μL methanol–water/min). Maximum centrifugal forces are given. Spot tests for amino acids were performed by treating dried applications of sample solutions on filter paper with ninhydrin reagent (0.25% in acetone) and heating at 100 °C for a few minutes.[54] DL-Serine was obtained from Sigma-Aldrich (Oakville, ON).

4.2. HPLC determination of amino acids

The utilization of amino acids in culture fluids was monitored by HPLC after derivatization using *o*-phthalaldehyde and mercaptoethanol, whereas derivatives formed using *o*-phthalaldehyde and *N*-acetyl-L-cysteine were used for the determination of enantiomeric compositions by HPLC.[36] Enantiomeric excesses (% ee) were calculated from peak areas corrected for the different fluorescent response of each enantiomer (i.e., peak area of D-enantiomer = 1.06 × peak area of the L-enantiomer), determined using triplicate injections of racemic serine at two different concentrations.

4.3. Microorganism, general growth conditions, and media

Fusobacterium nucleatum (ATCC 25586) was maintained and cultured at 37 °C under anaerobic conditions as described.[36] Liquid cultures in the peptone medium[36] were initiated with *F. nucleatum* cells grown for 24 h on a 9-cm sheep-blood agar plate and suspended in sterile peptone medium (3 mL).

The buffer for resuspension experiments[41] contained (g/L): K_2HPO_4, 9.0; KH_2PO_4, 6.0; $MgSO_4{\cdot}7H_2O$, 0.2; $CaCl_2{\cdot}2H_2O$, 0.02; sodium acetate, 0.3; and L-cysteine·HCl, 0.4. DL-Serine was dissolved in freshly prepared resuspension buffer, and the pH was adjusted to 7.3–7.4 with 3 M NaOH. The solution was autoclaved and supplemented with vitamin solution 1 (10 mL/L), vitamin solution 2 (1 mL/L), and trace metal solution (1 mL/L), each autoclaved separately. Vitamin solution 1 contained *p*-aminobenzoic acid, thiamin chloride hydrochloride, riboflavin, nicotinic acid, pyridoxal hydrochloride, inositol, calcium pantothenate, each at 0.2 g/L, and was adjusted to pH 7.0 with 1 M NaOH. Vitamin solution 2 contained DL-thioctic acid, biotin, haemin, folic acid, each at 0.1 g/L. The trace metal solution contained (g/L): $FeSO_4{\cdot}7H_2O$, 4.0; $MnSO_4{\cdot}2H_2O$, 0.15; and $Na_2MoO_4{\cdot}2H_2O$, 0.15.

4.4. Development of resuspension conditions

The peptone medium (500 mL) was inoculated with *F. nucleatum* cell suspension from one agar plate and incubated for 24 h. The damp cells (approximately 2.5 g) were harvested aseptically in air by centrifugation (8200*g*, 15 min) and resuspended with

gentle magnetic stirring in defined medium containing DL-serine (50 mL). Samples (300 μL) were removed at various times and centrifuged (15,400*g*, 10 min). Supernatants were stored at −15 °C for HPLC analysis.

4.5. Identification of metabolic end-products

The supernatant from a serine resuspension experiment (800 mM, 48 h incubation) was titrated to pH 9.5 with 5 M NaOH and lyophilized. A portion of the lyophilzed residue (ca. 100 mg) was dissolved in D_2O for NMR analysis. Resonances in the NMR spectra were assigned by chemical shift comparisons with standard samples. The relative amounts of the end-products were calculated from the integrated areas of the ^1H NMR signals for the methyl groups of acetate, butyrate and lactate at δ 1.84, 0.81 and 1.26 ppm, respectively.

4.5.1. Residue from serine catabolism

^1H NMR δ 4.04 (q, J = 6.9 Hz), 1.26 (d, J = 6.7 Hz) [lactate]; 2.08 (t, J = 7.3 Hz), 1.55–1.41 (m), 0.81 (t, J = 7.3 Hz) [butyrate]; 1.84 (s) [acetate]; 3.94–3.81 (AB of ABX, J_{AB} = 12.2 Hz), 3.74 (X of ABX, apparent t, splittings of 4.9 and 4.3 Hz) [serine]. ^{13}C NMR δ 185.3 (s), 71.2 (d), 22.8 (q) [lactate]; 184.2 (s), 26.0 (q) [acetate]; 175.9 (s), 63.2 (t), 59.1 (d) [serine]; 42.3, 22.0, 16.0 [butyrate].

4.6. Preparation of D-serine by cell suspensions

F. nucleatum resuspended in a phosphate buffer (5 g damp cells/100 mL) containing DL-serine (8.4 g, 800 mM) was incubated anaerobically with gentle magnetic stirring for 42 h at 37 °C and centrifuged (8200*g*, 15 min). The supernatant was applied to Amberlite IR-120 (2.5 × 50 cm column, H^+ form) at a flow rate of 5.0 mL/min. The column was washed with water (500 mL) and eluted with 0.5 M aqueous ammonia (3 L). Fractions (200 mL) containing amino acid (ninhydrin spot test) were combined and evaporated to dryness in vacuo. The residue (approximately 3.5 g) was dissolved in hot water (100 mL), treated with decolorizing charcoal (2 g), and filtered through Celite. Concentration of the filtrate in vacuo and the addition of ethanol yielded D-serine (3.47 g, 83% recovery), mp 213–215 °C, $[\alpha]_D^{25} = -15.7$ (*c* 1, 1 M HCl), 98% chemical purity by HPLC, >99% ee by HPLC, Ref. 55 mp 217–219 °C, Ref. 56 $[\alpha]_D^{23} = -15.0$ (*c* 4, 1 M HCl). ^1H NMR δ 3.98–3.86 (overlapping AB quartets of ABX, J_{AB} = 12.2 Hz, 2H), 3.80 (X of ABX, apparent t, splittings of 4.9 and 4.3 Hz, 1H). ^{13}C NMR δ 175.1, 62.8, 59.0; ESI^+MS (MeOH–H_2O, 1:1, 20 μL/min) m/z 106 $[M+H]^+$; CID of m/z 106: m/z 89 (2), 88 (26), 60 (100).

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