Growth of bacteria at low oxygen concentrations

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Modelling of Mixed Chemostat Cultures of an Aerobic Bacterium, *Comamonas testosteroni*, and an Anaerobic Bacterium, *Veillonella alcalescens*: Comparison with Experimental Data

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A mathematical model of mixed chemostat cultures of the obligately aerobic bacterium *Comamonas testosteroni* and the anaerobic bacterium *Veillonella alcalescens* grown under dual limitation of l-lactate and oxygen was constructed. The model was based on Michaelis-Menten-type kinetics for the consumption of substrates, with noncompetitive inhibition of *V. alcalescens* by O2. The growth characteristics of the aerobic and anaerobic organisms were determined experimentally with pure cultures of the individual species in (oxygen-limited) chemostats. Using these pure-culture data in the model of the mixed culture resulted in a good description of the actual mixed cultures of the two bacteria. In the actual mixed-culture experiments, coexistence of the two species occurred only when the cultures were oxygen limited. With increasing oxygen supply (the actual oxygen concentration in the culture remaining at <0.2 μM), the biomass of *C. testosteroni* increased, whereas that of *V. alcalescens* decreased. Apparently, *C. testosteroni* protected *V. alcalescens* from inhibition by oxygen by maintaining sufficiently low oxygen concentrations. The model calculations indicated that competition between the aerobic and anaerobic bacterium for common substrates (l-lactate and oxygen) occurred and that the anaerobe was the better competitor. Analysis of the culture fluid indicated that *C. testosteroni* grew primarily at the expense of the fermentation products of *V. alcalescens*, i.e., propionate and acetate. The model further indicated that different values of several growth parameters (e.g., substrate affinity and/or inhibition constants), the affinity of the aerobic organism for oxygen and the sensitivity of the anaerobic organism for oxygen were the most important properties determining the coexistence of these two physiologically different types of bacteria.

Microbial ecosystems with aerobic and anaerobic zones are found in many environments. Examples of this are sediments (16, 31, 32), microbial mats (33), soils (39), (stratified) lakes and seas (1, 22, 35, 40), biofilms (25), periodontal pockets (20), the rumen (37), and bacterial colonies (42). At the interface of aerobic and anaerobic zones, the concentration of dissolved oxygen is often very low but still sufficient for the growth of aerobes (9) and not high enough for total inhibition of anaerobes. Recently, we showed that strictly aerobic and anaerobic bacteria can grow side by side under microaerobic conditions in chemostat cultures provided the supply of oxygen does not exceed the maximum O2 consumption rate of the bacteria (8). Under such oxygen-limited conditions, fermentative, sulfate-reducing, and even very oxygen-sensitive methanogenic bacteria have been shown to coexist with obligately aerobic bacteria (8, 11, 13, 26). The anaerobic bacteria were apparently protected against O2 damage thanks to the high oxygen uptake affinity of the (facultively) aerobic bacteria ensuring a very low dissolved-oxygen concentration (3, 4, 34). The aerobes consumed the end products of the metabolism of the anaerobic bacteria.

To further investigate the environmental conditions and growth characteristics which may influence the coexistence of aerobes and anaerobes, we studied mixed cultures of the anaerobic fermentative bacterium *Veillonella alcalescens* and the aerobic bacterium *Comamonas testosteroni* as an experimental model system. In preliminary experiments, these two bacteria were shown to coexist over a considerable range of oxygen supply rates in chemostat cultures grown under dual limitation of l-lactate and oxygen (8).

Under anaerobic conditions, l-lactate is fermented by *V. alcalescens* to propionate, acetate, H2, and CO2. Although oxygen can be reduced by *V. alcalescens*, it also inhibits the growth of this anaerobe. The oxygen metabolism of *V. alcalescens* in pure culture has been studied extensively by De Vries et al. (7). It was demonstrated that this bacterium can grow in the presence of oxygen when the concentration of O2 remains less than approximately 0.8 μM. This study further revealed that in the presence of oxygen, *V. alcalescens* is able to consume l-lactate via a membrane-bound lactate oxidase system, resulting in the production of superoxide anion radicals and hydrogen peroxide. Although catalase activity was detected in *V. alcalescens*, inhibition of growth of this organism by oxygen was correlated with the accumulation of toxic oxygen metabolites in the medium and a rapid (reversible) inactivation of lactate dehydrogenase.

The strictly aerobic strain of *C. testosteroni* used in the present study was enriched selectively in a microaerobic chemostat (partial O2 pressure of <1 μM) with l-lactate as the carbon and energy source (8). In addition to l-lactate, the two major fermentation products of *V. alcalescens* (propionate and acetate) can be used by *C. testosteroni*. Hydrogen could not be metabolized.

The main objective of this study was to explore in more detail the coexistence of *V. alcalescens* and *C. testosteroni* at various degrees of nutrient availability. To this end, a mathematical model of this mixed culture was constructed to obtain a better understanding of the changes in the fluxes of substrates and fermentation products in response to a changing supply of oxygen and growth substrates. With the

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L-Lactate, propionate, and acetate can be

...detected in our laboratory as described before (8, 17). The bacteria were grown at 30°C in a phosphate-buffered minimal medium (pH 7.0) with L-lactate, propionate, or acetate as a carbon source in chemostats as described previously (8). Oxygen concentrations in the culture liquid and in the headspace were measured continuously with polarographic oxygen probes (Ingold). Redox readings were obtained continuously with a platinum electrode and with the Ag-AgCl reference electrode of the pH electrode as the reference cell (8). The chemostat was operated at a dilution rate of 0.1 h⁻¹.

Analytical procedures. Gases (H₂, CO₂, and O₂), total organic carbon, and maximum oxygen consumption rates of cell suspensions were determined as described before (8). Oxygen consumption rates in chemostat cultures were measured continuously by oxygen gas analysis of the in- and the outflowing gas (air-N₂ mixtures) (8). Organic acids were measured by gas chromatography using the method of Nanninga and Gottschal (23). Protein was quantified according to the method of Lowry et al. with bovine serum albumin as the standard (21).

Determination of kinetic parameters. Kinetic parameters for L-lactate, acetate, propionate oxidation, and oxygen consumption by C. testosteroni and V. alcalescens were determined respirometrically at 30°C in a biological-oxygen monitor (Yellow Springs Instruments Co., Yellow Springs, Ohio) equipped with a polarographic oxygen electrode. At very low oxygen concentrations, the electrical current measured at the output of the oxygen electrode was amplified 10⁰ times with a Keithley model 485 picoamperometer. Cells were taken from the chemostat, washed, and suspended in a phosphate buffer (50 to 250 mg of cell protein · liter⁻¹) containing chloramphenicol (30 mg · liter⁻¹) to block protein synthesis during the incubations (8). Initial maximum oxygen consumption rates of the resuspended cells did not differ much (<15% difference) from those obtained with cells transferred directly from the chemostat into the biological-oxygen monitor. To determine the half saturation constants for the consumption of growth substrates, cell suspensions were saturated with air and subsequently supplied with L-lactate, propionate, or acetate in limiting concentrations (5 to 50 μM). To determine the kinetics for oxygen consumption, these substrates were added at saturating concentrations (2 mM). The bacteria used in these incubations were pregrown on L-lactate or on a mixture of L-lactate, propionate, and acetate in an oxygen-limited chemostat. O₂ consumption rates were calculated as the tangent of O₂ concentration versus time plots at different substrate concentrations. Apparent Kₚ and Qₘₐₓ (rate of consumption at saturating concentration of substrate; see below) values for L-lactate, acetate, and propionate were estimated by the direct linear-plot method (27). Kinetic parameters for anaerobic L-lactate consumption by V. alcalescens were determined by directly measuring L-lactate depletion in an anaerobic batch culture. In all oxygen uptake experiments, dilute cell suspensions were used (5 to 30 mg of protein · liter⁻¹) to minimize interference of membrane O₂ diffusion characteristics with O₂ consumption. This allowed accurate measurement of oxygen concentrations of ≥0.01 μM. It was assumed that air-saturated buffer contained 250 μM dissolved O₂.

Mathematical model. The model is based on the assumption that substrate-dependent growth of bacteria in mixed culture can be described according to the same mathematical formulations as are used for the description of growth in pure cultures (10, 12, 18, 19, 28, 38, 41; see below also). The model was designed according to the following three basic steps.

(i) Step I. Determine the specific rates of consumption (Q) of the various substrates by the bacteria. This was done according to Michaelis-Menten-type kinetics.

\[
Q = Q_{\text{max}} \times s / (s + K_{\text{m}})
\]  

(1)

In this equation, \(Q_{\text{max}}\) represents the value of \(Q\) at a saturating concentration of \(s\) (\(s \gg K_{\text{m}}\)), \(s\) is the substrate concentration, and \(K_{\text{m}}\) is the half saturation constant.

(ii) Step II. Calculate the corresponding specific growth rates (\(\mu\)) of the individual organisms. The following equation was used.

\[
\mu = Q \times Y
\]  

(2)

In this equation, \(Y\) is the amount of biomass formed per substrate consumed (yield coefficient).

(iii) Step III. Determine the changes in the concentrations of L-lactate, oxygen, propionate, acetate, and biomass in the chemostat. This was done according to standard differential equations describing growth in continuous culture (see below). For explanation of the symbols and their default values, see Table 1.

Growth of C. testosteroni in the mixed culture is influenced by the availability of three potential carbon substrates, i.e., acetate, propionate, and L-lactate, and by the presence of oxygen (Fig. 1). V. alcalescens is also capable of reducing oxygen. Its specific growth rate is to some extent inhibited by O₂, depending on the actual concentration of O₂. Growth in the presence of very low dissolved-oxygen concentrations results in a change in the relative amounts of

![Diagram](image-url)
products formed during l-lactate fermentation and in the molar growth yield (7; this study).

The respiration rates of C. testosteroni (QO₂\textsubscript{Com} and V. alcalescens (QO₂\textsubscript{Veil}) are composed of the combined activities of two enzyme systems, one with high affinity and one with much lower affinity (see Results). Thus, for C. testosteroni, the specific oxygen consumption rate was described as follows.

\[ QO₂\textsubscript{Com} = (QO₂\textsubscript{max,1} \times [O₂]/[O₂] + K_{oxygen,1}O₂\textsubscript{Com}) + (QO₂\textsubscript{max,2} \times [O₂]/[O₂] + K_{oxygen,2}O₂\textsubscript{Com}) \]

A strictly analogous description is used for oxygen consumption by V. alcalescens.

The specific l-lactate consumption rate of C. testosteroni is expressed as follows.

\[ QL\textsubscript{Com} = QL\textsubscript{max,1} \times L/[L + K_{l-lactate,1}\textsubscript{Com}] \]

The specific propionate and acetate consumption rates of C. testosteroni and the l-lactate consumption rate of V. alcalescens determined either anaerobically or aerobically were formulated analogously.

The specific substrate consumption rates were used to calculate the specific growth rates (\(\mu\)) of the bacteria in the culture by using the following procedure.

(i) Determine the actual oxygen consumption rates by the populations of C. testosteroni and V. alcalescens in the culture.

To this end, the specific substrate consumption rates were multiplied by the respective biomass concentrations and an oxygen consumption factor (\(F\)) representing the fraction of the oxygen supplied to the culture that was consumed by the individual species C. testosteroni and V. alcalescens. This fraction was determined by dividing the sum of the specific substrate consumption rates of one of the populations in the culture by the sum of these rates for the total culture. For example, oxygen consumption by the population of C. testosteroni in the culture becomes

\[ \nuO₂\textsubscript{Com} = QO₂\textsubscript{Com} \times \alpha\textsubscript{Com} \times F\textsubscript{Com} \]

For oxygen consumption by the population of V. alcalescens, a strictly analogous equation was used.
D) (11)

(ii) Determine the actual consumption rates of carbon sources by the C. testosteroni and V. alcalescens populations in the culture.

L-Lactate, propionate, and acetate consumption by C. testosteroni is proportional to the amount of O₂ consumed by this bacterium. L-Lactate consumption \( v_{L^{\text{Com}}} \) by C. testosteroni in the culture was calculated by multiplying \( v_{O_{2}^{\text{Com}}} \) by the molar amount of L-lactate used per mole of oxygen consumed \( (\text{MOL}^{\text{Com}}) \) and by the fraction of the O₂ consumed by C. testosteroni \( (FL) \) for the oxidation of this substrate. This \( O_{2} \)-L-lactate consumption fraction was determined by dividing the specific consumption rate of \( L \)-lactate by the sum of the specific consumption rates of \( L \)-lactate, propionate, and acetate.

\[
v_{L^{\text{Com}}} = v_{O_{2}^{\text{Com}}} \times \text{MOL}^{\text{Com}} \times FL \quad (6)
\]

Rates of propionate and acetate consumption by C. testosteroni were calculated analogously.

(iii) Calculate the specific growth rates of C. testosteroni and V. alcalescens at the expense of the individual substrates.

The specific growth rates were calculated according to equation 2. For example, for growth of C. testosteroni on L-lactate, the following equation was used.

\[
\mu_{L^{\text{Com}}} = v_{L^{\text{Veil}}} \times \text{YL}^{\text{Com}} \quad (8)
\]

At growth-limiting substrate concentrations, it is reasonable to assume that the specific growth rate of an organism corresponds to the sum of the specific growth rates at the expense of the individual substrates \((10, 12)\). Thus, the specific growth rate of C. testosteroni becomes

\[
\mu_{C. \text{testosteroni}}^{\text{Com}} = \mu_{L^{\text{Com}}} + \mu_{P^{\text{Com}}} + \mu_{A^{\text{Com}}} \quad (9)
\]

where \( \mu_{C. \text{testosteroni}}^{\text{Com}} \) is \( \leq 0.66 \text{ h}^{-1} \), the maximum specific growth rate of C. testosteroni in an aerobic batch culture on a mixture of L-lactate, propionate, and acetate.

Inhibition of the growth of V. alcalescens by \( O_{2} \) was assumed to be noncompetitive. Thus, the specific growth rate of V. alcalescens was expressed as follows.

\[
\mu_{V. \text{alcalescens}} = (v_{L^{\text{Veil}}} \times \text{YL}^{\text{Veil}} / [1 + (\text{O}_{2} / K_{O_{2}^{\text{Veil}}})]) \quad (10)
\]

where \( v_{L^{\text{Veil}}} \times \text{YL}^{\text{Veil}} \) is \( \leq 0.3 \text{ h}^{-1} \), the maximum specific growth rate of V. alcalescens on L-lactate in an anaerobic batch culture. In equation 10, \( K_{O_{2}^{\text{Veil}}} \), the inhibition constant, represents the oxygen concentration at which \( \mu^{\text{Veil}} \) equals \( 0.5 \times \mu_{\text{max}}^{\text{Veil}} \).

Changes in the densities of V. alcalescens and C. testosteroni in the chemostat were calculated on the basis of the following general differential equation.

\[
dx/dt = x \times (\mu - D) \quad (11)
\]

The changes in substrate and product concentrations in the culture were calculated as follows.

\[
dS/dt = \text{supply to the culture} - \text{outflow by dilution} - \text{consumption by V. alcalescens} - \text{consumption by C. testosteroni}
\]

The primary growth substrate, L-lactate, was supplied to the chemostat from the reservoir medium \((i.e., D \times L_{0})\).

Supply of propionate and acetate was through the fermentation of L-lactate by V. alcalescens \( (v_{L^{\text{Veil}}}) \). Because the stoichiometries of the formation of propionate and acetate from L-lactate by V. alcalescens depended on the amount of \( O_{2} \) the organism consumed, these were determined experimentally in pure cultures of V. alcalescens at different rates of aeration \((\text{this study})\). The \( H_{2} \) and biomass production rates of V. alcalescens in the mixed culture were calculated on the basis of the same experiments.

Oxygen supply rates in the range of 0.0 to 3.6 mM \text{ h}^{-1} \ were used in the various mixed-culture simulations.

The differential equations were solved numerically by using the method described by Van der Hoef and Gotschel \((41)\). This provided automatic adjustment of the time intervals \((dt)\) to avoid negative substrate concentrations. The computer program was written in Turbo Pascal version 4.0 and can be executed on IBM-compatible computers containing the 8087 mathematical coprocessor. The model can be adapted relatively easily to describe mixed cultures of other aerobic and anaerobic bacteria growing under oxygen limitation.

**RESULTS**

Experiments with pure cultures of V. alcalescens and C. testosteroni were performed to obtain information on the essential growth parameters of these bacteria under oxygen-limited conditions.

**Growth of C. testosteroni on L-lactate, propionate, and acetate in batch and O₂-limited continuous cultures.** During growth of C. testosteroni under oxygen limitation in the chemostat, the actual oxygen concentration in the culture remained below the detection level of the electrode \((<0.2 \mu \text{M})\). The molar growth yield on L-lactate under these conditions was calculated for different oxygen supply rates in the range of 0.0 to 3.6 mM \text{ h}^{-1} \ and can be executed on IBM-compatible computers containing the 8087 mathematical coprocessor. The model can be adapted relatively easily to describe mixed cultures of other aerobic and anaerobic bacteria growing under oxygen limitation.

**Modelling of mixed chemostat cultures**

**TABLE 2. Analysis of chemostat cultures of C. testosteroni grown at various oxygen supply rates on various substrates**

<table>
<thead>
<tr>
<th>Steady state no.</th>
<th>Redox reading (mV)</th>
<th>O₂ \text{Lactate}</th>
<th>Prop</th>
<th>Acet</th>
<th>CO₂</th>
<th>Pyr</th>
<th>Cell C</th>
<th>Carbon recovery (%)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>34</td>
<td>1.43</td>
<td>0.91</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.30</td>
<td>0.06</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>1.24</td>
<td>0.27</td>
<td>0.40</td>
<td>0.13</td>
<td></td>
<td></td>
<td>1.62</td>
<td>0.12</td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>2.15</td>
<td>0.50</td>
<td>0.54</td>
<td>0.28</td>
<td></td>
<td></td>
<td>1.62</td>
<td>0.16</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>2.57</td>
<td>0.69</td>
<td>0.63</td>
<td>0.53</td>
<td></td>
<td></td>
<td>2.43</td>
<td>0.00</td>
</tr>
</tbody>
</table>

* Substrates were L-lactate (steady state 1; \( S_{L} = 20.0 \text{ mM} \)) and a mixture of L-lactate, propionate, and acetate (steady states 2, 3, and 4; \( S_{L} = 7.0 \text{ mM each} \) as the carbon and energy source \((D = 0.1 \text{ h}^{-1})\).

* Abbreviations: Lact, L-lactate; Acet, acetate; Prop, propionate; Pyr = pyruvate; Cell C, cell carbon.

* Expressed as grams of cell carbon produced per mole of carbon substrate consumed.

* CO₂ analysis not reliable.
conditions was 15.4 g of cell carbon per mol of L-lactate consumed (Table 2, steady state 1), which is very similar to that obtained in an oxygen-sufficient batch culture (15.1 g·mol⁻¹). From aerobic batch cultures grown on propionate and acetate, yield values of 18.8 and 10.6 g·mol⁻¹, respectively, were obtained. Growth yields of C. testosteroni in statically incubated (O₂-limited) batch cultures were significantly lower than those in well-aerated cultures. Values of 11.7, 15.3, and 8.7 g/mol of L-lactate, propionate, and acetate, respectively, were obtained. On the basis of these yield values, the amounts of substrate used per mole of oxygen consumed appeared to be 0.5 (l-lactate), 0.5 (propionate), and 0.8 (acetate) mol/mol.

The maximum specific growth rate of C. testosteroni in a shake culture on l-lactate or propionate was 0.54 h⁻¹. The maximum growth rate on L-lactate was 0.43 h⁻¹. On a mixture of l-lactate, propionate, and acetate, the organism grew at a slightly higher rate of 0.66 h⁻¹. When grown in spent supernatant of an L-lactate-grown culture of V. alcalescens, C. testosteroni had a growth rate of 0.43 h⁻¹ at the expense of propionate and acetate formed during growth of V. alcalescens.

Hydrogen was not oxidized by C. testosteroni in batch cultures incubated under an air-H₂ headspace in the presence or the absence of l-lactate.

Oxygen-limited chemostat cultures of C. testosteroni grown on a mixture of L-lactate, propionate, and acetate (7 mM each in the reservoir medium) at different oxygen supply rates used all three substrates simultaneously. However, L-lactate and propionate were consumed to a larger extent than acetate (Table 2, steady states 2, 3, and 4). Similar results were obtained with O₂-limited and O₂-sufficient batch cultures grown on mixtures of these substrates (data not shown). Small amounts of pyruvate were excreted by C. testosteroni grown under oxygen limitation in continuous culture (Table 2).

Growth of V. alcalescens in batch and aerated continuous cultures. V. alcalescens NS.L49 was grown in an l-lactate-limited chemostat (D = 0.1 h⁻¹) at different rates of oxygen supply. The anaerobe consumed the O₂ that entered the culture liquid, and steady states were obtained at O₂ consumption rates of 0.00, 0.35, and 0.70 mM·h⁻¹ (Table 3). Aeration of the culture resulted in an increase of the concentrations of relatively oxidized fermentation products (CO₂ and acetate) and a decrease of more-reduced products (H₂ and propionate). The molar growth yield increased significantly with the higher O₂ supply rates. On the basis of these steady-state data, the formation of fermentation products by V. alcalescens was described as a function of the amount of oxygen consumed (Fig. 2). Second-order polynomials were used to fit data on the fermentation pattern and growth yield of V. alcalescens to curves describing the response to increased oxygen consumption. The equations of these curves were used subsequently to approximate the changes anticipated in the oxygen-limited mixed cultures.

The maximum specific growth rate of V. alcalescens on l-lactate in an anaerobic batch culture was 0.30 h⁻¹, which increased slightly (to 0.34 h⁻¹) if measured in spent supernatant of a culture of C. testosteroni after reaeration of 20 mM l-lactate.

Growth of V. alcalescens on pyruvate was analyzed because C. testosteroni produced pyruvate from l-lactate when this organism was grown under oxygen limitation. In an anaerobic batch culture with pyruvate as the carbon and energy source, V. alcalescens grew at a rate of 0.12 h⁻¹. Compared with the results of growth on l-lactate, the products were more oxidized. One mole of pyruvate was fermented to 0.14 mol of propionate, 0.68 mol of acetate, 0.39 mol of H₂, 0.68 mol of CO₂, and 0.32 mol of cell carbon. The growth yield was 3.9 g of cell carbon·mol of pyruvate⁻¹.

Estimation of substrate consumption kinetics. Kinetics for the consumption of oxygen and the oxidation of l-lactate,
Modelling of mixed chemostat cultures

FIG. 3. Respirometric determination of the kinetic parameters for acetate consumption. (A) The change in oxygen concentration upon the addition of 25 μM acetate was traced until the respiration rate had returned to the endogenous rate. (B) The change in the concentration of acetate during the oxygen uptake experiment was calculated from the tracing in panel A. (C) The rate of acetate consumption was plotted as a function of the acetate concentrations as obtained by measuring the tangent of the substrate depletion plot (panel B) at different times. (D) A direct linear plot was constructed from the rate-versus-concentration data obtained from panel C. In this case of acetate consumption, a $K_m$ of 4.3 μM and a $Q_{max}$ of 4.2 μmol·mg of cell carbon·h$^{-1}$ were obtained.

Acetate, and propionate were determined respirometrically with resting-cell suspensions of *C. testosterone* and *V. alcalescens*. An example of the determination of the kinetic parameters for acetate consumption by *C. testosterone* grown on a mixture of l-lactate, propionate, and acetate in an O$_2$-limited chemostat is shown in Fig. 3. An oxygen tracing (Fig. 3A) from which an acetate depletion curve is obtained (Fig. 3B) is subsequently converted into a graph representing the acetate consumption rates at different acetate concentrations (Fig. 3C). From such graphs, the maximum specific consumption rate ($Q_{A_{max}}$) and the half saturation constant ($K_m$ for acetate) were estimated by using a direct linear plot (Fig. 3D).

The half saturation constants for the consumption of l-lactate, propionate, and acetate by *C. testosterone* and *V. alcalescens* were very similar, ranging from 4.3 to 6.0 μM (Table 1). The half saturation constant of *V. alcalescens* for l-lactate oxidation, as determined in the biological-oxygen monitor (5.1 μM), was essentially the same as that measured directly as l-lactate depletion in an
Estimation of the oxygen inhibition constant of *V. alcalescens*. Simulation of a pure culture of *V. alcalescens* yielded an accurate description of the experimentally observed growth pattern (i.e., steady-state fermentation product and biomass concentrations), provided that the O₂ supply rate remained between 0.0 and 0.5 mM · h⁻¹ and the oxygen inhibition constant for growth of *V. alcalescens* (Kₒ, O₂,m⁰) was >6 µM. At O₂ supplies of >0.5 mM · h⁻¹ or with O₂ inhibition constants of <6 µM, *V. alcalescens* could not establish a steady state in the chemostat. Fig. 6 shows the relationships among the specific growth rate of *V. alcalescens*, the inhibition constant for oxygen, and the actual oxygen concentration in the culture. In the mixed-culture simulations, a value of 10 µM was used.

Modelling of mixed cultures of *V. alcalescens* and *C. testosteroni* and comparison with experimental observations. A mathematical model of the mixed culture of *V. alcalescens* and *C. testosteroni* that used the experimentally determined growth parameters (Table 1) predicted steady states in which the two organisms coexisted as long as oxygen remained limiting, i.e., insufficient for *C. testosteroni* to oxidize all available carbon sources in the culture. The predicted steady-state biomass of *C. testosteroni* and *V. alcalescens* in the chemostat (Fig. 5A) as well as the production rates of propionate and acetate (Fig. 5B) calculated by the model were quite similar to those observed experimentally. However, the simulated H₂ production rates were somewhat higher than those observed in the chemostat studies. Although the dissolved-oxygen concentration remained below the detection limit of the oxygen probe (<0.2 µM) in the experimentally obtained steady states, the model predicted concentrations of ≥0.5 µM in these mixed cultures.

The model did predict coexistence of both species at higher oxygen supply rates than were obtained in the published experiments on which the model was based. The biomass of *C. testosteroni* increased linearly with the amount of oxygen supplied to the culture. Increased l-lactate concentrations in the reservoir medium resulted in an increase in *V. alcalescens* both in the chemostat experiments.
Modelling of mixed chemostat cultures

FIG. 5. Model predictions and experimental results of mixed cultures of *V. alcalescens* and *C. testosteroni* at various rates of oxygen supply. (A) Steady-state concentrations of biomass of *V. alcalescens* and *C. testosteroni* and dissolved oxygen in an l-lactate-limited chemostat (*D* = 0.1 h⁻¹, *S₅* = 19.1 mM l-lactate). Open symbols (with error bars representing standard deviations) represent the measured cell densities (expressed as cell carbon) of *V. alcalescens* (○) and *C. testosteroni* (●). Closed symbols represent the concentrations predicted by the model, using the experimentally determined default parameters given in Table 1. Oxygen concentrations predicted by the model are also shown (△).

(B) Measured (open symbols) and predicted (closed symbols) formation rates of propionate (△), acetate (○), and H₂ (●) by the mixed-chemostat culture of *V. alcalescens* and *C. testosteroni* grown on l-lactate and subjected to several oxygen supply rates. (C) Model calculations of the carbon substrate consumption rates in steady states of mixed cultures of *C. testosteroni* and *V. alcalescens*. l-Lactate consumption by *V. alcalescens* (+) and *C. testosteroni* (●), propionate consumption by *C. testosteroni* (●), and acetate consumption by *C. testosteroni* (○) are shown. (D) Model calculations for oxygen consumption rates by the individual bacteria *V. alcalescens* (●) and *C. testosteroni* (○) in the mixed-culture steady states.

and in the simulations (not shown). This clearly demonstrated that the mixed cultures were growing under a dual limitation of l-lactate and oxygen.

For completely anaerobic conditions, the model predicted a complete washout of *C. testosteroni* and the establishment of a monoculture of *V. alcalescens* (Fig. 5A). Also, at very low oxygen supply rates (<0.025 mM h⁻¹), the model predicted that *V. alcalescens* would completely outcompete *C. testosteroni*. Under such conditions, *V. alcalescens* consumed all l-lactate and oxygen supplied to the culture. However, at higher rates of oxygen supply, most of the oxygen was consumed by *C. testosteroni* (Fig. 5D) and most of the l-lactate was consumed by *V. alcalescens* (Fig. 5C), resulting in stable coexistence. Increasing oxygen supply rates resulted in increasing oxygen, l-lactate, propionate, and acetate consumption by *C. testosteroni* and decreasing l-lactate consumption by *V. alcalescens*. Only after most of the propionate and acetate had been used did *C. testosteroni*
consume a substantial part of the l-lactate supplied to the culture. Together with the increasing level of oxygen (Fig. 5A), this resulted in a decreasing steady-state biomass concentration of \( V. \) alcalescens. At oxygen supply rates above 3.6 mM \( \cdot \) h\(^{-1}\), the anaerobe washed out of the culture and a steady-state pure culture of \( C. \) testosteroni with l-lactate as the limiting substrate, oxygen in excess, and a biomass concentration of 17.5 mM cell carbon was obtained.

**Effects of changes in parameter values of the model.** To study the importance of the various growth parameters for coexistence of \( V. \) alcalescens and \( C. \) testosteroni, the outcome of the model with the measured parameter values (Table 1) at an \( O_2 \) supply rate of 2 mM \( \cdot \) h\(^{-1}\) was compared with the predicted outcome following a change of one of these parameter values. With a 10-fold increase or decrease in the carbon substrate consumption parameters \( (Q_{\text{max}} \text{ or } K_v) \text{ values}) \) or yield coefficients \( (Y) \) of \( V. \) alcalescens or \( C. \) testosteroni, the model still predicted coexistence of the two bacteria but with altered steady-state substrate and biomass concentrations. However, when the \( O_2 \) consumption capacity of \( C. \) testosteroni was reduced by decreasing the \( Q_{\text{O}_2,\text{max}} \text{Consum} \) from 22.4 to <15 \( \mu \text{mol} \cdot \text{mg} \text{ of cell carbon}^{-1} \cdot \text{h}^{-1} \) or by increasing the \( K_{\text{O}_2,\text{Consum}} \) from 0.35 to >5 \( \mu \text{M} \), coexistence was no longer predicted. With these parameter values, the calculated dissolved-oxygen concentration increased to values (>20 \( \mu \text{M} \)) which repressed the specific growth rate of \( V. \) alcalescens to a value below the dilution rate (<0.1 h\(^{-1}\)), which can be seen in Fig. 6. Obviously, coexistence also depended on the oxygen inhibition constant of \( V. \) alcalescens. When the \( K_{\text{O}_2,\text{Yield}} \) was reduced from 10 to <0.5 \( \mu \text{M} \), \( V. \) alcalescens could no longer maintain itself in the culture.

**DISCUSSION**

The aim of this study was to investigate the interactions between the strictly anaerobic \( V. \) alcalescens and the obligately aerobic \( C. \) testosteroni, which in a preliminary study (8) were shown to coexist in chemostats operated under dual limitation of oxygen and l-lactate. To this end, a mathematical model was constructed to describe the experimental mixed cultures in more detail, thus helping us understand the observed pattern of growth in response to various degrees of oxygen supply. The current model is a further elaboration of earlier models that is based on Monod-type growth kinetics and is used to describe both pure and mixed continuous cultures (10, 12, 19, 38, 41).

A basic assumption common to most of these models is that the specific growth rate (\( \mu \)) can be described as a saturating function of the growth substrate concentration (\( S \)) with a constant maximum specific growth rate (\( \mu_{\text{max}} \)) and a half saturation constant (\( K_S \)): the familiar Monod equation, \( \mu = \mu_{\text{max}} \cdot S/(K_S + S) \).

Significant modifications of this equation are necessary if more than one substrate simultaneously limits growth of an organism. One way of doing this is by summation of the specific growth rates on the individual substrates by using the Monod equation (10). However, an important assumption to be made is that each individual substrate does not affect the kinetics for growth on the other compounds. This assumption could not be made for the \( C. \) testosteroni strain used in the present mixed-culture experiments, as it appeared that under oxygen-limiting conditions, l-lactate and propionate were used preferentially and very little acetate was consumed (see also below). Therefore, the present model was based on the measured values of the kinetic parameters for substrate consumption, which together determine the specific growth rate. These parameters could be obtained in pure cultures of \( V. \) alcalescens and \( C. \) testosteroni grown in oxygen-limited chemostats in the presence of the potential substrates in the mixed culture. It was then assumed that these substrate consumption kinetics were not significantly different from those in the mixed cultures. The observation that the maximum specific growth rates of \( C. \) testosteroni in spent supernatant of \( V. \) alcalescens and of \( V. \) alcalescens in spent supernatant of \( C. \) testosteroni were close to the rates in freshly prepared media provided strong support for the validity of this assumption.

**Pure-culture observations.** To determine the substrate consumption kinetics of \( V. \) alcalescens and \( C. \) testosteroni, pure cultures of these bacteria were grown at a dilution rate of 0.1 h\(^{-1}\) under oxygen limitation with either l-lactate alone or a mixture of l-lactate, propionate, and acetate as growth substrate.

Steady states of l-lactate-limited pure cultures of \( V. \) alcalescens were obtained at several rates of \( O_2 \) supply with the \( O_2 \) concentration in the culture liquid remaining <0.2 \( \mu \text{M} \). The consumption of oxygen by \( V. \) alcalescens caused a change in the fermentation pattern from that of strictly anaerobic cultures, resulting in the formation of more relatively oxidized products (acetate and \( \text{CO}_2 \)) and less relatively reduced products (propionate and \( \text{H}_2 \)). The increased acetate formation was paralleled by a significant increase in the molar growth yield on l-lactate that presumably was due to increased ATP formation in the metabolic route via acetyl-phosphate to acetate. Several examples of organisms responding in a similar way have been reported for the genera *Lactococcus*, *Selenomonas*, *Propionibacterium*, *Bacteroides*, and *Actinomyces* (2, 5, 6, 30, 36).
Pyruvate, a major end product found by De Vries et al. (7) in cultures of V. alcalescens exposed to oxygen concentrations higher than those used in this study (0.8 to 250 μM), was not produced in the steady-state cultures of our strain. The V. alcalescens strain used in the present study appeared to use pyruvate as a growth substrate.

When grown on a mixture of l-lactate, propionate, and acetate under oxygen limitation, C. testosteroni consumed all of these substrates simultaneously, but acetate was used at a significantly lower rate. Pyruvate was not detected in the mixed-culture steady states, indicating that V. alcalescens may have consumed it. However, pyruvate production by C. testosteroni and subsequent consumption by V. alcalescens were not included in the mathematical model of such mixed cultures, since it would have caused only minor changes in the predictions of the model.

Measuring the potential substrate-oxidizing capacities by respirometry proved useful for estimating kinetic parameters for the consumption of substrates and oxygen (18). The oxidation of all three carbon substrates (l-lactate, propionate, and acetate) became the only substrate of the anaerobic cultures, since it would have caused only minor changes in the predictions of the model.

Pyruvate was used to test the rate at which oxygen consumption of V. alcalescens are much higher than those of C. testosteroni. This results in a concentration of oxygen below the value needed by C. testosteroni to sustain a specific growth rate equal to the dilution rate. Thus, the paradoxical situation occurs in which an anaerobe outcompetes an aerobic for oxygen. Experimental data from mixed cultures are not available to substantiate this theoretical prediction, since oxygen supply rates below 0.025 mM·h⁻¹ were not tested.

At oxygen supply rates for which coexistence was predicted, it appeared that most of the l-lactate was used by V. alcalescens and that C. testosteroni grew predominantly at the expense of the fermentation products of V. alcalescens, propionate and acetate. So, V. alcalescens appeared the better competitor for l-lactate, even under conditions of microaerobic growth. These considerations explain why the biomass of V. alcalescens in the mixed-culture steady states did not vary much, whereas that of C. testosteroni increased linearly with increasing oxygen supply.

Changing the growth parameters of the two species used in the model revealed which parameters were most significant in determining coexistence of the two organisms. It appeared that coexistence is ruled predominantly by the inhibition of V. alcalescens by oxygen in combination with the high O₂ consumption capacity of C. testosteroni, a situation very similar to that encountered in mixed cultures of Actinomyces viscosus and Streptococcus mutans grown under oxygen-limiting conditions (41). Coexistence of these organisms under a dual limitation of oxygen and glucose depended on consumption of oxygen by A. viscosus and inhibition of the growth of S. mutans by oxygen. In general, continuous growth of an anaerobe in an aerated chemostat is possible only if the consumption of oxygen by bacteria present in the culture is sufficient to ensure a dissolved-oxygen concentration low enough to allow for a specific growth rate exceeding the dilution rate of the culture. Of course, the oxygen-consuming bacteria may be the (oxygen-tolerant) anaerobic organisms themselves, or they may be accompanying facultatively aerobic bacteria.

The results of this study demonstrate that the combined use of chemostat experiments and a mathematical model can help us understand interactions between aerobic and anaerobic bacteria and better define the conditions allowing such organisms to grow as stable mixed cultures.

Further investigations of species with other characteristics (e.g., anaerobes more sensitive to oxygen than V. alcalescens) are essential for obtaining full confidence in the use of such models and for evaluating the importance of these results for the growth of various aerobic and anaerobic bacteria in the environment.

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REFERENCES


33
Chapter 3


