Elevated precursor 16S rRNA levels suggest the presence of growth inhibitors in wastewater

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Abstract Conventional activated sludge systems require bacteria to grow to avoid washout through decay and routine solids wasting. Recently we developed a procedure targeting precursor 16S ribosomal RNA to measure the *in situ* growth activity of phylogenetically defined microbial populations, and this procedure was used to study the growth of bacteria in activated sludge systems. The current study significantly expands this previous work by quantifying levels of precursor 16S ribosomal RNA within individual cells of pure cultures of bacteria exposed to various culture conditions. Initially, three ranges (Type I, Type II, and Type III) of precursor 16S ribosomal RNA levels were defined by whole cell fluorescence *in situ* hybridization of a pure culture of *Acinetobacter calocaceticus*^{T} prepared in three culture conditions. Low levels of precursor 16S ribosomal RNA (Type I) corresponded to a stationary phase culture prepared overnight in Luria-Bertani medium. Intermediate levels of precursor 16S ribosomal RNA (Type II) corresponded to a culture transferred into fresh Luria-Bertani medium, and high levels of precursor 16S ribosomal RNA (Type III) corresponded to a culture treated with the growth inhibiting antibiotic chloramphenicol. Subsequently, the abundance of individual cells of each Type were measured in four different pure cultures after exposure to 0.45-μm filtered primary effluent collected from four different conventional activated sludge treatment plants in Cincinnati, OH, USA. Individual cells of each Type were observed in the culture of *A. calcoaceticus*^{T} exposed to each of the four primary effluents. Only Type I cells were observed in cultures of *A. johnsonii*^{T}, *A. johnsonii* strain 210a, and *Escherichia coli*^{T} exposed to each of the four primary effluents. These results suggest that the growth of *A. calcoaceticus*^{T} was inhibited by an unidentified component of filtered primary effluent present in each of the four wastewaters; whereas the growth of *A. johnsonii*^{T}, *A. johnsonii* strain 210a, and *E. coli*^{T} were not inhibited. These results have significance for understanding the growth of phylogenetically defined microbial populations within activated sludge treatment systems. If the pattern of elevated p16S rRNA levels observed in *A. calcoaceticus*^{T} is prevalent in many microbial populations in activated sludge systems, this may have implications for preventing washout of critical microbial populations that may be experiencing growth inhibition.

Keywords *Acinetobacter*; activated sludge; *in situ* growth activity; precursor 16S rRNA; ribosome genesis

Introduction

Conventional activated sludge (AS) systems require growth of bacteria in order to avoid washout due to decay and routine wasting of sludge from secondary clarification. The wasting rate, solids levels, and mass balance equations often are used to estimate the net growth rate of the composite AS biomass (i.e. Activated Sludge Model No. 1, Henze *et al*., 1987). Respirometry and kinetic studies of substrate utilization are used to determine the net growth rate of metabolically defined microbial populations (i.e. nitrifiers or phosphorus accumulating organisms) (Grady *et al*., 1996). Recently, molecular-biology-based methods for studying microbial communities have been used to measure the *in situ* growth activities of phylogenetically defined microbial populations in a variety of environments including wastewater treatment systems (Pollard *et al*., 1998; Oerther *et al*., 1999; Molin and Givskov, 1999).

One promising approach for measuring *in situ* growth activity involves ribosome genesis. It is well documented that the production of ribosomes, also known as ribosome

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genesis, shows a strong positive correlation with the growth of pure cultures of microorganisms in laboratory conditions (reviewed in Bremer and Dennis, 1996). During normal growth of bacteria, precursor 16S ribosomal RNA (p16S rRNA) serves as a key intermediate in ribosome genesis (reviewed in Jemiolo, 1996). The rRNA genes are transcribed as a polycistron containing the 16S, 23S, and 5S rRNA as well as a variety of transfer RNAs. RNAse III releases the p16S rRNA from the polycistron. The p16S rRNA is subsequently converted into mature 16S rRNA through the action of a number of parallel processing pathways. Some antimicrobial agents, including chloramphenicol and derivatives, inhibit ribosome genesis and the growth of bacteria by selectively inhibiting the maturation of p16S rRNA.

Cangelosi and Brabant (1997) used membrane and sandwich hybridizations to measure p16S rRNA levels and mature 16S rRNA levels in *Escherichia coli* exposed to various growth, nutritional, and inhibitory conditions. The levels of p16S rRNA increased more than 50-fold when a stationary phase culture of *E. coli* was transferred to fresh nutrient rich medium and dropped more than 25-fold when the culture entered stationary phase. Carbon and nitrogen limitation resulted in distinct decreases in the levels of p16S rRNA. The addition of the antibiotic rifampin to a rapidly growing culture inhibited transcription resulting in a reduction in the levels of p16S rRNA, while chloramphenicol addition interfered with p16S rRNA processing halting the decrease of p16S rRNA during starvation.

In a similar study, Licht and coworkers (1999) employed whole-cell fluorescence *in situ* hybridizations (FISH) to compare the levels of p16S rRNA within individual cells of *E. coli* growing in two distinct intestinal compartments of a mouse. The caecal mucus layer contained fast growing *E. coli* with corresponding low levels of p16S rRNA, while the caecum extract inhibited growth of *E. coli* and corresponded to high levels of p16S rRNA. Licht and coworkers hypothesized that the high levels of p16S rRNA suggested the presence of an unidentified inhibitory substance in the caecum of the mouse. They subsequently tested a known growth inhibitor, chloramphenicol, and observed the same high levels of p16S rRNA in single cells of *E. coli*. This result was consistent with the observation of Cangelosi and Brabant (1997) and suggested that high levels of p16S rRNA were an indication of the presence of unidentified growth inhibiting compounds in the caecum of the mouse.

Previously, we have demonstrated that transient changes in the levels of p16S rRNA in *Acinetobacter calcoaceticus*^{T} are a sensitive measure of *in situ* growth activity in pure cultures as well as in laboratory- and full-scale municipal wastewater treatment systems (Oerther *et al*., 2000; Oerther *et al*., 2002; Oerther and Raskin, 2001). In addition, we observed that individual cells of *A. calcoaceticus*^{T} contained variable levels of p16S rRNA when they were exposed to primary effluent collected from a municipal wastewater treatment plant (Oerther *et al*., 2000).

In the current study, we hypothesize that the variable levels of p16S rRNA observed in individual cells of *A. calcoaceticus*^{T} are an indication of variable growth rates amongst individual cells of single bacterial populations in AS systems. To test this hypothesis, we measured the levels of p16S rRNA in a pure culture of *A. calcoaceticus*^{T} exposed to various growth conditions. Furthermore, we measured the levels of p16S rRNA in pure cultures of *A. johnsonii*^{T}, *A. johnsonii* strain 210a, and *E. coli*^{T} exposed to the same growth conditions.

Materials and methods

Pure cultures

A. calcoaceticus^T (American Type Culture Collection strain 23055), *A. johnsonii*^{T} (ATCC 17909), *A. johnsonii* strain 210a (Deinema *et al*., 1980), and *E. coli*^{T} (ATCC 11755) were

maintained on Luria-Bertani (LB) medium (per litre of water: 10 g tryptone, 5 g yeast extract, 10 g NaCl) and LB agar (per litre of LB medium: 15 g agar).

Preparing *A. calcoaceticus*^{T}

A. calcoaceticus^T was cultured overnight in LB medium at 20°C on a rotary shaker. A 5-ml aliquot of the overnight culture was transferred into 95 ml of fresh LB medium. After 60 min, chloramphenicol was added to the inoculated culture to achieve a final concentration of 20 mg/litre. Samples of *A. calcoaceticus*^{T} were removed from the overnight culture and from the fresh culture 15 min after inoculation and 60 min after the addition of chloramphenicol.

Wastewater samples

Effluent from the primary clarifier was collected at four conventional AS wastewater treatment plants in Cincinnati, OH, USA. The four plants included two plants treating domestic wastewater, the Muddy Creek Wastewater Treatment Plant and the Indian Creek Wastewater Treatment Plant, and two plants treating municipal wastewater containing a heavy industrial contribution, the Little Miami Wastewater Treatment Plant and the Mill Creek Wastewater Treatment Plant. The four samples were vacuum filtered through 0.4-μm pore size Gelman filters. Filtered samples were analyzed for pH and ammonia using an Orion model 720A meter and a Ross pH probe (model 8102) and an Orion ammonia probe (model 95-12). Nitrate, nitrite, and chemical oxygen demand (COD) were determined using an appropriate Test 'N Tube procedure (Hach, Loveland, CO).

Exposing bacteria to wastewater

The four pure cultures, *A. calcoaceticus*^{T}, *A. johnsonii*^{T}, strain 210a, and *E. coli*^{T}, were cultured overnight in LB medium at 20°C on rotary shakers. The cultures were equilibrated at 35°C for 1 h and each culture was diluted twenty-fold into each of the four filtered primary effluents. The sixteen dilutions were incubated at 35°C using rotary shakers. Every hour, a 2 mL-sample was removed from each dilution and stored for further analysis.

Fluorescence *in situ* hybridization

The samples were fixed for 1 h at room temperature in 4% (wt/vol) paraformaldehyde prepared in 1x phosphate buffered saline pH 7.0 (1 × PBS is 130 mmol l^{-1} NaCl and 10 mmol l^{-1} sodium phosphate buffer). Subsequently, the samples were stored in a 50% (vol/vol) mixture of ethanol and 1 × PBS pH 7.0 at –20°C. The fluorescently-labeled oligonucleotide probes used in this study included: S-D-Bact-0338-a-A-18 (5′ GCTGCCTCCCGTAGGAGT 3′) labeled with Cy5 or FITC (Amann *et al*., 1990); S-S-Acin-1543-a-A-24 (5′ GATTCTTACCAATCGTCAATCTTT 3′) labeled with Cy3 (Oerther *et al*., 2000); and S-S-E.coli-1543-b-A-24 (5′ GCACTACAAAGTACGCTTCTTTAA 3′) labeled with Cy3 (Oerther *et al*., 2000). Fixed samples were applied in a sample well on a Heavy Teflon Coated microscope slide (Cel-Line Associates, New Field, NJ) and air-dried. After dehydration with an increasing ethanol series (50, 80, 95% [vol/vol] ethanol, 1 min each), each sample well was covered with 9 μL of hybridization buffer (20% [vol/vol] formamide, 0.9 mol l^{-1} NaCl, 100 mmol l^{-1} Tris HCl [pH 7.0], 0.1% SDS) (de los Reyes *et al*., 1997). Fluorescently labeled oligonucleotide probe, 1 μL (50 ng), was added to each well of the microscope slide and hybridizations were conducted in a moisture chamber for 1 h, in the dark, at 46°C. The slides were washed for 30 min at 48°C with 50 mL of prewarmed wash solution (215 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris HCl [pH 7.0], 0.1% SDS, and 5 mmol l^{-1} EDTA) (de los Reyes *et al*., 1997). Samples were counter stained with ice-cold, fresh 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) staining solution (0.2 mg/litre) for 1

min, rinsed with water, and rapidly air-dried. Fixed, hybridized cells were mounted with Cargille immersion oil (Type FF, Cedar Grove, NJ) and a cover slip.

Individual cells were visualized and the optimum focus for the microscope was achieved using the DAPI filter set. The FITC-labeled probe was visualized before the Cy3-labeled probe. These procedures were used to help minimize photobleaching of the Cy3, Cy5, and FITC fluorescent labels. Probe conferred fluorescence was visualized with a Nikon Microphot epifluorescence microscope, and digital images were captured using a 12-bit gray-scale for a Spot-2 charge coupled device (CCD) camera. Identical signal capture protocols were used for each field. Digital images were manipulated using MetaMorph (version 4.5, Universal Imaging Corp., Downington, PA) imaging software and Adobe Photoshop (version 6.0, Adobe Systems Incorporation, San Jose, CA).

The digital images for each sample were analyzed in the following manner. First, a binary mask to identify objects (one or more cells) was generated using the results from the S-D-Bact-0338-a-A-18 probe. This mask was then applied to the image of the appropriate p16S rRNA probe. This procedure reduced the non-specific p16S rRNA probe signal and sample artifacts. Objects with sizes smaller than a typical microbial cell were eliminated using a size mask. The average object gray value was determined for the p16S rRNA probe signal for individual objects. Ten images for each sample were analyzed in this manner.

Results and discussion

Defining three types of p16S rRNA levels

Previously, we observed that exposure to filtered primary effluent resulted in variable levels of p16S rRNA amongst individual cells of a pure culture of *A. calcoaceticus*^{T} (Oerther *et al.*, 2000). Furthermore, the results suggested that three subpopulations of individual cells could be identified based upon distinct ranges of p16S rRNA levels. To develop defined ranges of p16S rRNA levels corresponding to specific growth conditions, samples were removed from a pure culture of *A. calcoaceticus*^{T} exposed to three growth conditions. One sample was removed from an overnight culture of *A. calcoaceticus*^{T} (condition 1, O/N). A second sample was removed from a culture of *A. calcoaceticus*^{T} after 15 min of exposure to fresh LB growth medium (condition 2, O/N+LB), and a third sample was removed from a culture of *A. calcoaceticus*^{T} after 60 min of exposure to the growth inhibiting antibiotic chloramphenicol (condition 3, O/N+LB+C). FISH was conducted using fluorescently labeled oligonucleotide probes targeting mature 16S rRNA and p16S rRNA. Hybridization signals for the mature 16S rRNA-targeted probe were used to identify individual objects, and the fluorescence signal intensity of the p16S rRNA-targeted probe for each object was quantified using semi-automated digital image analysis. The results of fluorescence signal intensity for the p16S rRNA-targeted probe are presented in Figure 1.

The average fluorescence intensity for objects for growth condition 1 were between 800 and 1,600 gray-scale units. Condition 2 had a range of fluorescence signals from 850 to 2,100 gray-scale units, and condition 3 had a range of fluorescence signals from 1,050 to 2,950 gray units. Approximately one-half of the objects for condition 2 had gray-scale values higher than the maximum gray-scale values of objects in condition 1. More than one-half of the objects for condition 3 had gray-scale values higher than the maximum gray-scale values of objects in condition 2. These results were used to define three ranges of p16S rRNA levels corresponding to three Types of growth conditions for individual bacteria cells (Table 1). Type I corresponded to low growth activity typical of overnight cultures, and the range of fluorescence signal intensity for p16S rRNA-targeted FISH was defined as 750–1,600 gray-scale units per object. Type II corresponded to stimulated growth activity typical of cultures transiently exposed to improved growth conditions including fresh growth medium. Gray-scale values per object from 1,601 to 2,100 units corresponded to

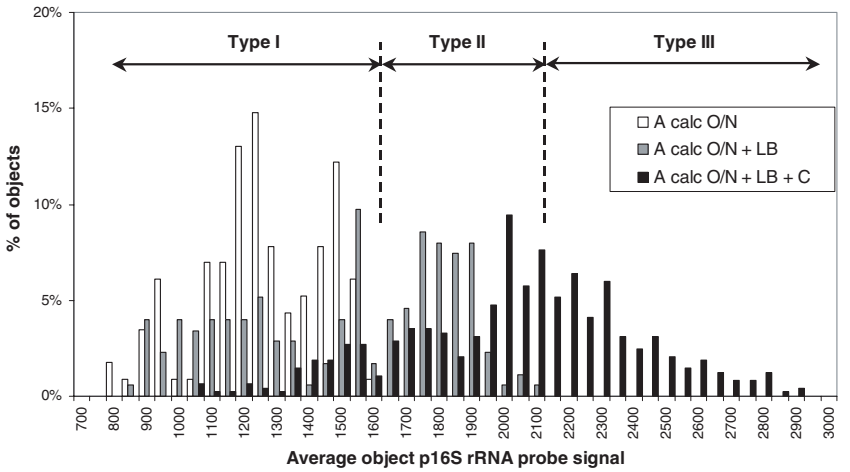

Figure 1 Histogram of the average object gray level of the p16S rRNA probe signal from fluorescence *in situ* hybridizations for three culture conditions of *A. calcoaceticus*^{T}. The white bars represent condition 1 (O/N), the gray bars represent condition 2 (O/N+LB), and the black bars represent condition 3 (O/N+LB+C). The p16S rRNA probe signal ranges corresponding to the three Types of p16S rRNA levels, Type I, II, and III, are also shown

Table 1 Cumulative percentage of objects with the three distinct ranges of p16S rRNA probe signal for the three *A. calcoaceticus*^{T} culture conditions

		A. calcoaceticus^{T} culture condition		
		1	2	3
	p16S rRNA probe signal range	O/N	O/N+LB	O/N+LB+C
Type I	750–1,600	100%	55%	14%
Type II	1,601–2,100	0%	45%	46%
Type III	2,101+	0%	0%	40%

Type II p16S rRNA levels. Type III corresponded to growth inhibition typical of cultures exposed to compounds that inhibit ribosome genesis, and the fluorescence intensity of p16S rRNA-targeted FISH for Type III was defined as a gray-scale value higher than 2,101 units.

Table 1 also shows the relative abundance of individual objects of each Type observed in each of the three culture conditions. The O/N culture condition contained only Type I cells of *A. calcoaceticus*^{T}. The O/N+LB culture condition contained near-equal amounts of Type I and Type II objects. Thus, approximately one-half of the individual cells of an overnight culture of *A. calcoaceticus*^{T} demonstrated an increase in growth activity 15 min after inoculation into fresh LB medium. The addition of chloramphenicol (O/N+LB+C) stimulated almost one-half of the individual cells of *A. calcoaceticus*^{T} to have Type III p16S rRNA levels. Thus, the presence of inhibitors of ribosome genesis, and therefore inhibitors of growth activity, resulted in an increase in Type III cells. These results are consistent with our previous work (Oerther *et al.*, 2000; Oerther *et al.*, 2002; Oerther and Raskin, 2001) as well as with the studies of Licht *et al.* (1999).

Determining the abundance of each type of p16s rRNA level in pure cultures exposed to filtered primary effluent

After defining the range of the intensity of the p16S rRNA fluorescence signal for Type I, Type II, and Type III cells, this information was used to evaluate the exposure of four pure cultures of bacteria to filtered primary effluent collected from four conventional AS

wastewater treatment plants. The four pure cultures were prepared overnight in LB medium, diluted twenty-fold into filtered primary effluent, and samples were removed for FISH every hour. Representative digital micrographs of the results of FISH with fluorescently labeled oligonucleotide probes targeting mature 16S rRNA and p16S rRNA are shown in Figure 2. *A. calcoaceticus*^{T} was exposed to filtered primary effluent number one (FPE1). After 5 hours, Type I, Type II, and Type III cells could be detected (Figure 2a). Since Type I cells contained a low level of p16S rRNA, the fluorescence signal intensity for the Cy-3 labeled p16S rRNA-targeted probe (shown in red) was significantly less than the fluorescence signal intensity for the FITC labeled mature 16S rRNA-targeted probe (shown in green) resulting in the appearance of green cells in the representative color-merged image (Figure 2a). Type II and Type III cells had higher levels of p16S rRNA and therefore they appeared as dim red cells and bright red cells, respectively (Figure 2a). After 24 h, only Type I cells could be detected in samples removed from the same culture (Figure 2b).

Using FISH with p16S rRNA-targeted and mature 16S rRNA-targeted oligonucleotide probes, the p16S rRNA levels of individual cells of *A. calcoaceticus*^{T} exposed to filtered primary effluent collected from four wastewater treatment plants were measured (Table 2). Exposure of *A. calcoaceticus*^{T} to all four sewage samples resulted in the occurrence of at least 5% of total cells as Type I, Type II, and Type III. The presence of Type III cells in each culture suggested that an unidentified component of wastewater was responsible for inhibiting ribosome genesis in *A. calcoaceticus*^{T}. The results shown here with wastewater collected in Cincinnati, OH are consistent with previous work with wastewater collected in Urbana, IL (Oerther *et al*., 2000), suggesting that unidentified inhibitory components of wastewater may exist in many AS systems.

Type I cells with low levels of p16S rRNA, were observed in all twenty-four samples removed from the culture exposed to FPE1, but some of the samples removed from the cultures exposed to FPE2, FPE3, or FPE4 did not contain Type I cells (Table 1). Type II and Type III cells were more abundant and persisted for a longer time in FPE2 (13 h), FPE3 (15 h), and FPE4 (13 h) as compared to FPE1 (6 h) (Table 2). These results suggest a difference in the composition of FPE1 as compared to FPE2, FPE3, and FPE4. As shown in Table 3, the levels of ammonia, nitrite, nitrate, and total nitrogen (sum of ammonia, nitrite, and nitrate) were very different for FPE1 as compared to FPE2, FPE3, and FPE4. The levels of ammonia were the lowest and the levels of nitrite and nitrate were the highest in FPE1

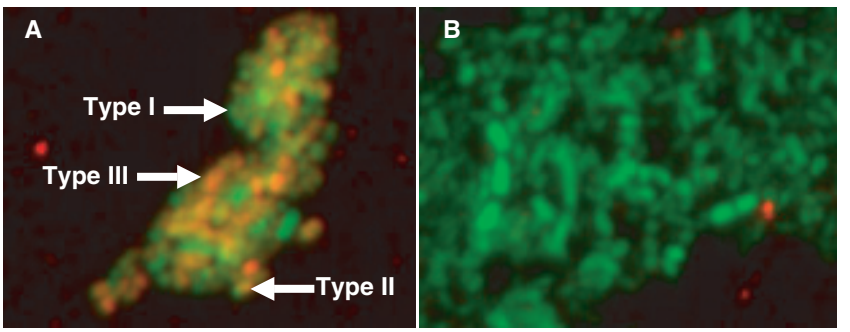

Figure 2 Digital micrographs of whole cell FISH of *A. calcoaceticus*^{T} with Cy3-labeled p16S rRNA probe (shown in red) and Cy5-labeled 16S rRNA probe (shown in green). *A. calcoaceticus*^{T} exposed to filtered primary effluent one for 5 h (A) and 24 h (B). The abundance of p16S rRNA in single cells of *A. calcoaceticus*^{T} are identified as Type I (green cells with low hybridization signal from the p16S rRNA-targeted fluorescently-labeled probe), Type II (dimly red cells with intermediate hybridization signal from the p16S rRNA-targeted probe), and Type III (bright red cells with strong hybridization signal from the p16S rRNA-targeted probe)

Table 2 Abundance of Type I, Type II, and Type III levels of p16S rRNA observed in a pure culture of *A. calcoaceticus*^{T} exposed for to filtered primary effluent collected from four wastewater treatment plants. Relative abundance above 5% of total cell counts is indicated by a positive sign

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
FPE1	**I**		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	II		+	+	+	+	+	+																	
	III				+	+	+	+																	
FPE2	**I**		+	+	+	+	+	+			+		+	+	+	+	+	+	+	+	+	+	+	+	+
	II		+	+	+	+	+	+	+	+	+	+	+	+	+										
	III		+	+	+	+	+		+	+	+		+	+											
FPE3	**I**		+							+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	II		+	+	+	+	+	+	+	+	+	+	+	+	+		+								
	III		+	+	+	+	+	+	+	+	+	+	+	+	+										
FPE4	**I**					+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	II		+	+	+	+	+	+	+	+	+		+		+										
	III		+	+	+			+	+				+												

(Table 3). A comparison of the results from the analysis of the composition of the filter primary effluents (Table 3) with the results from FISH with the p16S rRNA-targeted probe (Table 2) suggests that elevated levels of ammonia coupled with low levels of nitrite and nitrate corresponded to prolonged periods of Type II and Type III p16S rRNA levels. *A. calcoaceticus*^{T} has been shown to utilize both ammonia and nitrate as sources of assimilative nitrogen. Furthermore, it has been shown that *A. calcoaceticus*^{T} is not capable of growth via denitrification (Baumann *et al.*, 1968). Therefore, although the levels of reduced and oxidized nitrogen corresponded to extended periods of Type II and Type III cells, it is unclear whether the differences in nitrogen are responsible for the differences in p16S rRNA levels or if the differences in nitrogen are coincident with the differences in the p16S rRNA levels.

In contrast to the results observed when *A. calcoaceticus*^{T} was exposed to filtered primary effluent, the levels of p16S rRNA in pure cultures of *A. johnsonii*^{T}, strain 210a, and *E. coli*^{T} remained low throughout the 24-h incubation period and only Type I cells were observed (data not shown). These results suggest that the unidentified components of filtered primary effluent that inhibit ribosome genesis in *A. calcoaceticus*^{T} may not inhibit ribosome genesis in all bacteria.

Proposed model of the response of p16S rRNA in bacteria

Figure 3 presents a conceptual model of the response of p16S rRNA in bacteria. The model is based upon previous observations (Oerther *et al.*, 2000; Oerther *et al.*, 2002; Oerther and Raskin, 2001) as well as the information presented in this study. The model shows the changes in the levels of p16S rRNA in individual cells of a population of *A. calcoaceticus*^{T}

Table 3 Characteristics of primary effluent collected from two municipal and two domestic wastewater treatment plants in Cincinnati, OH. The location and type of wastewater treatment plant is specified. The levels of soluble chemical oxygen demand, ammonia nitrogen, nitrate nitrogen, nitrite nitrogen, and pH are provided

	FPE 1	FPE 2	FPE 3	FPE 4
Location	Little Miami	Mill Creek	Muddy Creek	Indian Creek
Treatment plant type	Municipal	Municipal	Domestic	Domestic
COD (mg/L)	72	287	52	85
NH_3 (mg-N/L)	9	53	15	32
Nitrate (mg-N/L)	43.9	0.9	0.9	0.9
Nitrite (mg-N/L)	2.3	0.2	0.2	0.2
pH	7.62	7.40	7.66	8.11

cultured overnight and transferred into fresh medium (Figure 3A), cultured overnight and transferred into fresh medium with subsequent addition of chloramphenicol (Figure 3B), and cultured overnight and transferred into filtered primary effluent (Figure 3C). Previous work with pure cultures of *A. calcoaceticus*^{T} demonstrated that exposure of overnight cultures to fresh Luria-Bertani medium produced a transient response in all individuals in the culture from Type I to Type II p16S rRNA levels (Figure 3A) (Oerther *et al*., 2000). In addition, cultures prepared in minimal medium with acetate as the sole carbon source demonstrated a similar transient response in all individuals in the culture (Oerther and Raskin, 2001). When cultures of *A. calcoaceticus*^{T} were exposed to chloramphenicol, the levels of p16S rRNA within individual cells increased to Type III in all individuals in the culture (Figure 3B, chloramphenicol addition indicated by the arrow) (Oerther *et al*., 2000). The response of *A. calcoaceticus*^{T} exposed to filtered primary effluent is shown in Figure 3C. In the overnight cultures, all of the individuals within the culture were Type I cells.

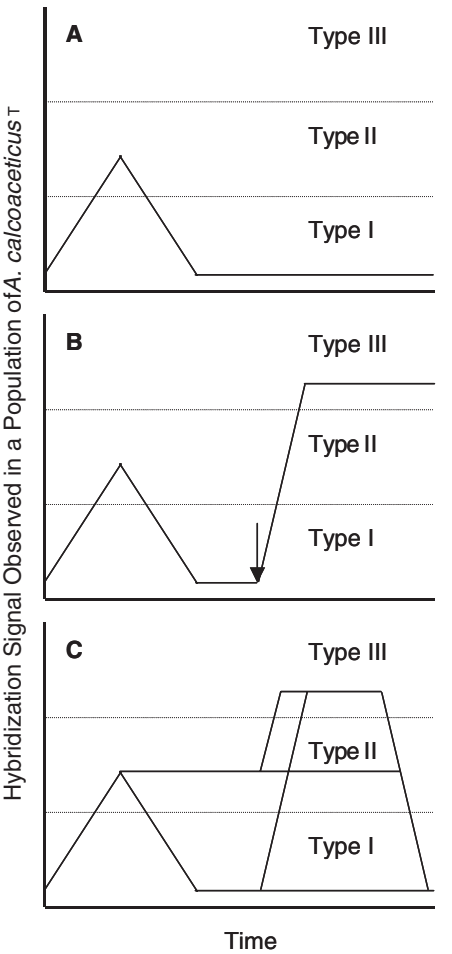

Figure 3 Schematic of the response of p16S rRNA abundance within individual cells of a population of *A. calcoaceticus*^{T} transferred to fresh medium (A), transferred into fresh medium with subsequent addition of chloramphenicol indicated by the arrow (B), and transferred into filtered primary effluent (C). Type I represents low levels of p16S rRNA typical of an overnight culture. Type II represents an intermediate level of p16S rRNA typical of the maximum level of the transient increase in the abundance of p16S rRNA levels observed in cultures exposed to fresh media. Type III represents high levels of p16S rRNA typical of cultures exposed to the antimicrobial agent chloramphenicol. Type III cells may be produced through an increase in p16S rRNA levels in Type II or alternatively Type I cells

Immediately following exposure to filtered primary effluent, the levels of p16S rRNA in most cells increased from Type I to Type II. Over time, some cells returned to Type I p16S rRNA levels and other cells increased from Type II p16S rRNA to Type III p16S rRNA levels. Alternatively, cells demonstrating Type I p16S rRNA levels may have transformed to Type III p16S rRNA levels. Eventually the levels of p16S rRNA in all individual cells of *A. calcoaceticus*^{T} returned to the Type I condition (Figure 2B and Figure 3C).

Conclusions

We have defined three Types of individual bacteria cells based on p16S rRNA levels that correspond to three growth conditions. These Type definitions were used to examine the response of pure cultures exposed to filtered primary effluent collected from four conventional AS wastewater treatment plants in Cincinnati, OH, USA. Exposure to the four wastewaters produced high levels of p16S rRNA (Type III) within individual cells of *A. calcoaceticus*^{T}. Although the growth of individual cells was not measured explicitly in this study, we speculate that the detection of Type III cells in *A. calcoaceticus*^{T} when exposed to wastewater is an indication of inhibited growth of a subpopulation of the culture. The presence of a growth-inhibited subpopulation could reduce the effective net growth rate of the overall population. Although only one of the four microbial populations tested showed this pattern of elevated p16S rRNA levels when incubated in primary effluent, the phylogenetic distribution of this observation is still in question. If the inhibition of ribosome genesis is prevalent in many microbial populations in AS systems, this may have implications for preventing washout of critical microbial populations that may be experiencing growth inhibition.

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