When Is a Microbial Culture "Pure"? Persistent Cryptic Contaminant Escapes Detection Even with Deep Genome Sequencing

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ABSTRACT ***Geobacter sulfurreducens*** **strain KN400 was recovered in previous studies in which a culture of the DL1 strain of *G. sulfurreducens* served as the inoculum in investigations of microbial current production at low anode potentials (−400 mV versus Ag/AgCl). Differences in the genome sequences of KN400 and DL1 were too great to have arisen from adaptive evolution during growth on the anode. Previous deep sequencing (80-fold coverage) of the DL1 culture failed to detect sequences specific to KN400, suggesting that KN400 was an external contaminant inadvertently introduced into the anode culturing system. In order to evaluate this further, a portion of the gene for OmcS, a *c*-type cytochrome that both KN400 and DL1 possess, was amplified from the DL1 culture. HiSeq-2000 Illumina sequencing of the PCR product detected the KN400 sequence, which differs from the DL1 sequence at 14 bp, at a frequency of ca. 1 in 10^5 copies of the DL1 sequence. A similar low frequency of KN400 was detected with quantitative PCR of a KN400-specific gene. KN400 persisted at this frequency after intensive restreaking of isolated colonies from the DL1 culture. However, a culture in which KN400 could no longer be detected was obtained by serial dilution to extinction in liquid medium. The KN400-free culture could not grow on an anode poised at −400 mV. Thus, KN400 cryptically persisted in the culture dominated by DL1 for more than a decade, undetected by even deep whole-genome sequencing, and was only fortuitously uncovered by the unnatural selection pressure of growth on a low-potential electrode.**

IMPORTANCE **Repeated streaking of isolated colonies on solidified medium remains a common strategy for obtaining pure cultures, especially of difficult-to-cultivate microorganisms such as strict anaerobes. The results presented here demonstrate that verifying the purity of cultures obtained in this manner may be difficult because extremely rare variants can persist, undetectable with even deep genomic DNA sequencing. The only way to ensure that a culture is pure is to cultivate it from an initial single cell, which may be technically difficult for many environmentally significant microbes.**

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Much of the progress in microbiology has depended on the study of "pure cultures." These are cultures that contain only one type of cell, ideally with the culture derived from an initial single cell. From the early development of methods for establishing pure cultures (1), there have been many examples in which cultures that were initially considered to be pure were subsequently found to contain two types of microbes. In some instances, these inadvertent multispecies cultures have led to important breakthroughs, such as the discovery of interspecies hydrogen transfer (2). However, with the advent of deep sequencing technologies, it might reasonably be predicted that undetected contaminants in cultures would no longer be a significant concern.

Geobacter sulfurreducens strain PCA was isolated in the mid-1990s by using standard enrichment and isolation techniques that included dilution to extinction in liquid medium, followed by repeated streaking of isolated colonies on agar-solidified medium (3). This is the classic strategy taught in the most popular introductory microbiology textbooks (4–6). Later, *G. sulfurreducens* strain DL1 was obtained by additional restreaking of isolated PCA colonies (7). With the development of methods for the genetic manipulation of *G. sulfurreducens* (7) and the sequencing of its genome (8), *G. sulfurreducens* became the *Geobacter* species of choice for the study of the physiology and novel extracellular electron transfer properties of this environmentally important genus (9). Initial sequencing of the 16S rRNA gene in the culture (3), as well as sequencing of the genome first to 8-fold (8) and then to 80-fold (10, 11) coverage, indicated that the culture contained only one strain.

In an attempt to adaptively evolve DL1 to produce more current, it was inoculated into a bioelectrical system with a graphite anode poised at a potential (−400 mV versus Ag/AgCl) considered to be near the lower limit at which current generation from acetate would be possible (12). An isolate obtained from the anode biofilm, designated *G. sulfurreducens* strain KN400 (12), is superior to the inoculum strain in producing electrical current, and this superiority is attributed at least in part to its ability to produce more "microbial nanowires," electrically conductive protein filaments that exhibit metallic-like electrical conductivity (13, 14).

It was initially assumed that strain KN400 had accumulated one or more mutations that enhanced its capacity for extracellular

TABLE 1 Estimates of strain KN400 abundance in various cultures[a]

Approach	No. of sequences	
	DL1 or PCA	KN400
Sequencing assay		
DL1 culture	1.5×10^7	286
PCA culture ATCC 51573	3.2×10^7	52
DL1 additional restreaking	2.5×10^7	185
DL1 serial-dilution culture	3.6×10^7	0
qPCR assay		
DL1 culture	1.5×10^7	980
PCA culture ATCC 51573	4.6×10^7	12
DL1 additional restreaking	2.5×10^7	33
DL1 serial-dilution culture	6.4×10^7	Undetectable

[a] In the sequencing assay, a portion of the sequence of *omcS* was amplified from genomic DNA with primers omcSRT F and omcSI R (see Table S1 in the supplemental material), the PCR product was sequenced, and the KN400- and DL1-specific *omcS* sequences, which differed in 14 bp (see Fig S1 in the supplemental material), were quantified. In the qPCR approach, KN400 abundance was estimated with primers hisRT F and hisRT R (see Table S1), which amplify a gene for a sensor histidine kinase response regulator found in KN400, but not in DL1, and total cell abundance was estimated with primers omcSRT F and omcSRT R (see Table S1), which amplify a portion of the *omcS* sequence in both strains.

electron transfer. This would be analogous to the selection for mutant strains during adaptive evolution of strain DL1 for electron exchange with other organisms (11) or higher rates of Fe(III) oxide reduction (10). However, comparative genomics revealed that the genome sequence of strain KN400 contained more than 27,270 single nucleotide polymorphisms (SNPs) (15). A third of the genes had at least one nucleotide polymorphism, and a quarter had a polymorphism that affected the resulting protein sequence (15). Based on typical mutation rates of 6.3×10^{-9}/bp per generation (16), it would take more than 1,000 years to accumulate this many mutations in strain DL1. Furthermore, there are no orthologs in the DL1 strain for 139 of the open reading frames (ORFs) in the KN400 strain genome, most of which are most closely related to genes in phylogenetically diverse organisms (15).

These considerations and the fact that the ORFs unique to KN400 were not detected when the inoculum culture was resequenced (10, 11) led to the hypothesis that KN400 entered the bioelectrochemical system as a contaminant. To evaluate this possibility, the purity of the DL1 culture was further assessed with even higher sensitivity.

Both DL1 and KN400 contain *omcS*, a gene for an outer surface cytochrome that has one of the highest proportions of SNPs (36 SNPs/kb) between the two strains (15). This gene was amplified from the DL1 culture that had been used to inoculate the bioelectrochemical system, and the PCR products were sequenced with Illumina Hi-Seq 2000 (see Text S1 in the supplemental material). The KN400 sequence was detected, indicating that KN400 was present in the initial inoculum used for the anode selection study. However, of the $>10^7$ high-quality sequences recovered, only 286 were KN400 sequences versus 1.5×10^7 DL1 sequences (Table 1).

The relative abundance of the KN400 strain in this same culture was further evaluated by quantitative PCR (qPCR) with a primer set specific for a gene found only in KN400 (see Text S1 and Table S1 in the supplemental material) and a primer set that detected both KN400 and DL1 (see Table S1 in the supplemental material). The relative abundance of the KN400-specific sequence was similar to that of the KN400 *omcS* sequences determined by the sequencing approach (Table 1). Sequence analysis and qPCR assay of the PCA culture deposited at ATCC revealed the presence of KN400 at a similar low abundance (Table 1).

We attempted to remove the rare KN400 contaminant from

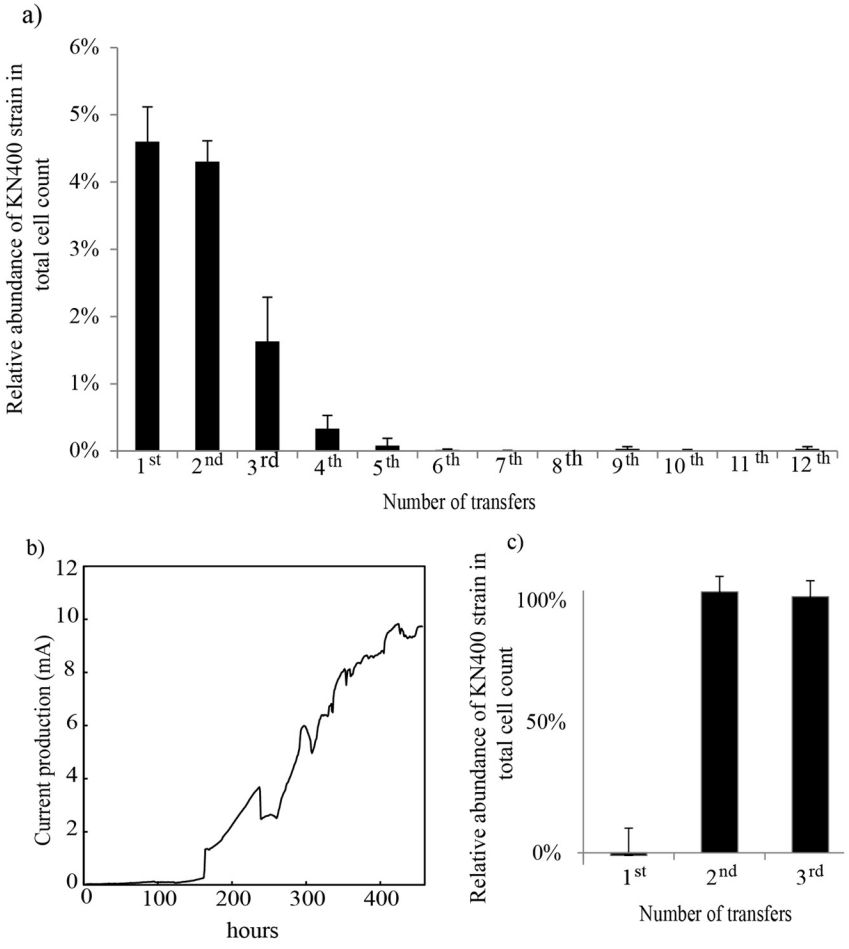

FIG 1 Growth and activity of strain KN400 under different growth conditions. (a) Relative abundance of KN400 in successive mid-log transfers (1% inoculum) of a culture initiated with equal proportions of KN400 and DL1. The results are the means and standard deviations of triplicate cultures. (b) Current production when the DL1 culture was introduced into the anode chamber of a bioelectrochemical system with a graphite anode poised at −400 mV versus Ag/AgCl. The KN400-free culture produced no current over this time. (c) Relative abundance of KN400 in anode biofilms when the initial inoculum was the DL1 culture subsequently found to also contain KN400. The results are the means and standard deviations of triplicate cultures.

the DL1 culture by repeated restreaking of isolated colonies grown on solidified acetate-fumarate medium (7), but the KN400 strain continued to persist at a low frequency in the isolated colonies (Table 1). However, a culture in which KN400 could no longer be detected was obtained in liquid acetate-fumarate medium (see Text S1 in the supplemental material) in which the highest dilution exhibiting growth was subjected to several successive rounds of serial dilution (Table 1). This demonstrated that the DL1 strain does not require the rare presence of KN400 in order to grow in the acetate-fumarate medium on which this culture is routinely maintained.

When pure KN400 and the KN400-free DL1 culture were inoculated into acetate-fumarate medium in equal quantities, DL1 outcompeted KN400 (Fig. 1a). This is consistent with the finding that when the two strains were grown separately, DL1 grew much faster than KN400 (optical density at 600 nm [OD_{600}] of 0.04 for KN400 versus 0.65 for DL1 after 36 h of incubation; see Fig. S2 in the supplemental material) in the same medium. The proportion of KN400 continued to decline with consecutive transfers (1% inoculum) until the abundance of KN400 was comparable to that in the PCA and DL1 stock cultures (see Text S1 in the supplemental material). KN400 persisted at this low level with further transfers (Fig. 1a).

Repeated attempts to grow the KN400-free DL1 culture on an anode poised at −400 mV were unsuccessful. However, current was readily produced in another set of experiments in which the DL1 culture containing KN400 served as the inoculum (Fig. 1b). A qPCR assay of DNA extracted from the anode biofilm indicated that 100% of the *omcS* sequences were KN400 within the second transfer (Fig. 1c). These results suggested that the reason why KN400 emerged on the anodes was that the DL1 strain was not able to grow under the conditions imposed. Without this unusual selective pressure, KN400 would have remained undetected in the PCA and DL1 cultures even by currently available next-generation sequencing methods that can theoretically provide thousandsfold coverage in genome sequencing.

The factors contributing to the long-term persistence of strain KN400 at extremely low frequency in the DL1 culture remain a mystery. The importance of physically isolating single cells by methods more definitive than streaking on solid medium to establish pure cultures has been recognized for some time (17), and increasingly more sophisticated methods for accomplishing this continue to be developed (18–23). However, the plating methods that have been in use for more than 100 years and are taught to every microbiology student as standard procedures for obtaining pure cultures (4–6) remain the most common. The results presented here demonstrate that without single-cell isolation, cryptic contaminants may survive at very low frequency in cultures over decades of intensive investigation and can escape detection by even the most sophisticated sequencing methods currently available.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00591-12/-/DCSupplemental.

Text S1, PDF file, 0.1 MB.
Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Table S1, PDF file, 0.1 MB.

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