Phylogenetic Characterization and Description of Novel Heat-tolerant *Bacillus* species Isolated from Spacecraft Assembly Facility

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Microbial biofilms representing both Gram-positive and Gram-negative bacteria were artificially coated onto aluminum metal surfaces that are chiefly used in building spacecraft. Changes in bacterial numbers were monitored over time on metal test pieces by conventional spread plate technique and polymerase chain reaction (PCR). Conventional standard plating technique did not recover desiccated bacterial biomass from either glass or aluminum metal surfaces even with a density of 10^6 cells per coupon. However, the whole-cell PCR (without extracting DNA) showed a positive signal for a density of 10^5 dehydrated microbial cells. Electron and epifluorescent microscopical examinations revealed that bacteria entered a nonculturable state once the water activity is reduced to minimum. However, a simple DNA extraction protocol combined with PCR amplification of specific 16S rDNA fragment improved the recovery of non-viable desiccated microbial biofilm from the metal pieces. In ongoing investigations to map and archive the microbial footprints in various components of spacecraft and its accessories, we have examined the microbial populations of the Spacecraft Assembly Facility (SAF). We have exposed witness plates that are made up of spacecraft materials and or painted with spacecraft-quality paints for ~7 to 9 months. In the initial studies reported here, we have examined the total cultivable aerobic heterotrophs. The results showed that the witness plates coated with spacecraft quality paints attracted more dust particles than the non-coated stainless steel witness plates. Among four paints tested, witness plates coated with NS43G (an off-white conductive paint [silicate binder]) accumulated the highest number of particles, hence attracted more cultivable microbes and spore-formers. The microbiological examination revealed that the SAF High Bay-1 harbors mainly Gram-positive microbes and mostly spore-forming *Bacillus* species. The phylogenetic relationships among these heat-tolerant microbes were examined using a battery of morphological, physiological, molecular and chemotaxonomic characterizations. Using phenotypic characterization, only 9 strains were identified. By 16S rRNA analysis, the isolates fell into seven clades: *Bacillus licheniformis, B. pumilus, B. cereus, B. circulans, Staphylococcus capitis, Planococcus* sp. and *Micrococcus lylae.*

The objectives of a planetary protection program for space missions involving the return of a sample from an extraterrestrial body to the Earth are: the absolute protection of the Earth from a possible biological hazard and forward contamination (relative protection of the extraterrestrial body from terrestrial origin). Forward contamination control for a Mars sample return mission has aspects of both forms of planetary protection. Mars must be protected from terrestrial contamination brought by space flight systems that contact it because Mars is an extraterrestrial body of scientific interest in the search for life. A stringent set of requirements is needed to assure the scientific integrity of the returned sample. The specific requirements for sample protection are complete sterility and surface cleanliness for particular hardware surfaces.

The prevention of organic contamination of the sample is a broader objective that is more difficult to achieve than the sterility requirement. Mere sterility permits non-viable microbes, fragments of microbes, organic substances of biological origin (*e.g.,* DNA, RNA, proteins, amino acids, etc.), and other organic compounds that may be mistaken as having biological origin. The contamination of the extraterrestrial sample with organic molecules must be avoided because detection strategies and high sensitivities might lead to false positives in life detection experiments.

In the past few years, due to the use of molecular methods, our knowledge of microbial diversity has increased dramatically, not only from a phylogenetic and taxonomic perspective but also from an ecological basis. We now know that microorganisms exist in every conceivable place on Earth. Temperature may be the only limitation as to where they can or cannot exist and/or function.

PCR is an *in vitro* nucleic acid amplification method. The reaction comprises repeated thermal cycling of the reaction mixture. The thermal cycling serves to dissociate the products of the previous thermal cycle then allow the association of these dissociated products with further reaction starting materials for another phase of synthesis. Specifically, substrate DNA is denatured at a high temperature to give single-stranded (template) molecules. This is followed by short oligonucleotide primers (amplimers) annealing to specific, nucleotide-sequence-defined regions of the template at a lower temperature. The positions where the amplimers anneal to the template define the "target". The thermal cycle is conducted by the amplimers being enzymatically extended by the coupling of appropriately base-paired deoxynucleoside triphosphates (dNTPs) on the template at an intermediate temperature, thus producing another double-stranded DNA (dsDNA) copy of the original target. More than a million-fold amplification in the copy number of a specific segment of DNA can be obtained in about 2 hours. Thus, beginning with only a trace amount of DNA, a sufficient detectable quantity can be generated so that detection can be accomplished with standard laboratory instruments.

The ribosome is a complex composed of individual RNA molecules and more than 50 proteins. The structure of the ribosomes is similar but not identical in all species. This consistency is another reflection of the common evolutionary origin of many of the most basic constituents of living cells. Ribosomal RNAs are at present the most useful and

most used in the molecular taxonomy. They occur in all organisms. They are large enough to get sufficient information for comparison. Today, molecular genetic analysis of the gene encoding 16S rRNA (rDNA) obtained from DNA extracted from various samples is routinely used and a broad range of otherwise similar strategies are applied without prior cultivation and isolation of the organisms. In principle, this technique in contrast to the standard NASA culturing techniques that rely on healthy, viable microbes that can grow on a particular set of nutrients, detects both viable, non-viable microbes and their remnants. In this report we have evaluated the efficacy of PCR technique in the detection of various microbes and compared their sensitivity with the standard NASA culturing techniques.

Microbes of concern in spacecraft assembly facility (SAF) are those that are commonly found in its territory (habitat-associated), those that indicate the sanitary or quality of hygienic practices (human-associated), and those that under certain conditions could cause human illness. Primary sources of microbial contamination include soil and dust, air, and earth which are very difficult to control. The secondary source includes air, handlers, cross-contamination, equipment, and buildings. Secondary sources of contamination are controlled by good manufacturing practices and standard industrial practices. The microbes of concern in SAF are primarily spore-forming bacteria and microbes that are indigenous to human skin, hair, and respiratory tract (Puleo et al., 1977). Bacterial spores, particularly those in the *Bacillus* genus, are common in the spacecraft industry.

In ongoing investigation to map and archive microbial footprints of various spacecraft components, we have examined the microbial populations of a SAF High Bay #1 at Jet Propulsion Laboratory (hereafter called as JPL-SAF). Witness plates from JPL-SAF were analyzed for contaminant composition and viability. The microbial population or diversity is an integral part of the ecosystem's function rather than a result of reacting to or upon ecosystem function. Since the microflora of SAF may have a direct impact on the quality of the product, the microbial diversity of this facility may give some insight and knowledge leading to the eradication of these microorganisms and improvements in the quality of the spacecraft being built.

MATERIALS AND METHODS

Comparison of conventional bioassay and molecular diagnosis in the detection of microbial population on metal surface

Bacterial strains. All bacteria were purchased from American Type Culture Collection. Bacterial cultures were grown in appropriate cultural conditions (media, temperature and duration) prior to use.

Microcosms A. Bacterial culture at log-phase were serially diluted using sterile distilled water as diluent to varying final concentrations (ranging from 0 to 10^9 cfu per mL). These microcosms are called as "initial inoculum".

Microcosms B. Glass or aluminum metal slides (5 x 7.5 cm rectangular size) were purchased from appropriate vendors. The glass and aluminum metal surfaces were cleaned with pure ethanol and individually wrapped with aluminum foil and autoclaved at 121°C for 30 min in a dry cycle. A 100 µl sample suspension (final concentrations ranging from 0 to 10^9 CFU per mL) was applied onto glass or metal surfaces in duplicate and allowed to dry at room temperature overnight. These microcosms are called "artificial biofilms". Sterile cotton swabs that were moistened in a portion of 1 mL of sterile distilled water were used to remove bacterial cells from the artificial biofilm of the surfaces as per NASA procedures. The swabbed bacterial cells were then transferred into the sterile phosphate buffered saline water for further analysis.

Total viable counts and viability assay. Suitable aliquots of various samples were spread plated onto respective agar plates and incubated at appropriate cultural conditions and colony forming units were enumerated. This is called as "total viable counts". Subsequently, an aliquot of sample was inoculated into liquid media and incubated at appropriate cultural conditions to check the sterility of the microcosms.

Polymerase chain reaction. In addition, a 10-µl sample suspension was used as template for the PCR assay without extracting DNA (whole-cell PCR). The DNA from the samples were also extracted as per established procedures (Johnson, 1981) and 10-µl purified DNA were used as template for PCR. The experimental conditions and primer selections for PCR are described in detail below. Suitable controls such as buffer, media, PCR reaction mixtures and DNA were employed to check any false-positive or false-negative reactions.

Microscopy. Suitable sample preparations were carried out to understand the morphology of the bacterial isolate before and after desiccation using various microscopy procedures. Samples were stained with BacLight stains to check the viability of the bacterial cultures by epifluorescene microscopy (Kepner et al., 1994). Scanning and transmission electron microscopic examinations were carried out as described in Cole and Popkin (1981). Further we have carried out *in situ* analysis using environmental scanning electron microscopy (ESEM; XL30; Philips) without going through any sample preparations. The elemental analysis was also carried out to know the chemical composition of the remnants or the intact cells.

Microbial characterization of Spacecraft Assembly Facility

Witness plates positioning and retrieval. The dimensions of the JPL-SAF are 80' wide, 120' long, and 44' 4" high. Relative humidity was controlled at 40 ± 5% with a cap at 45% and the average temperature was maintained at 20 ± 5°C. Entry into this facility was minimal and carefully monitored by setting a series of rigorous procedures.

Witness plates of a specified size (1"x2") were used. The pre-sterilized witness plates made of stainless steel and four different paint-coated aluminum plates were exposed in a

JPL spacecraft assembly facility. After the given exposure period, all witness plates were individually placed into 50-mL polypropylene disposable sterile centrifuge tubes.

Microbial examination of witness plates. Each witness plate retrieved from JPL-SAF was placed into sterile phosphate buffered rinse solution (Venkateswaran et al. 2000). The plate and rinse solution were sonicated for 2 min. One part of the rinse solution along with the witness plate were subjected to heat-shock (80°C for 15 min) and the other part was not heated. Two-mL aliquots were placed into sterile petri dishes in several replicates. The whole witness plate was also placed into a sterile petri dish. Molten Trypticase Soy Agar (TSA, Difco, Detroit, Michi.) was aseptically poured into these petri dishes containing samples and the contents were gently mixed to obtain uniform distribution of microbes. All plates were incubated in an inverted position at 32°C for 3 to 7 days and cfu were counted and recorded.

All strains were stained for Gram-reaction (Fisher Scientific Co., Atlanta, Georgia) and DAPI staining (Kepner et al. 1994). The ability to grow at various temperatures and sodium chloride concentrations were used as a discriminator to select strains. All the isolates that showed growth at 60°C were further characterized. However, some representatives of bacteria that were morphologically different but not growing at 60°C were also included.

Phenotypic characterization. Routine biochemical tests were carried out according to established procedures (Claus & Berkeley, 1986). The ability to grow at a NaCl concentration of 1 to 10% was determined in T_1N_1 liquid medium (Venkateswaran *et al.*, 1989), and the ability to grow without NaCl was determined in 1% sterile tryptone water. Temperature tolerance at 60°C was performed in TSB. The API CHB 50 kit was used for the Gram-positive spore-forming rods and the API STAPH kit was used for Gram-positive coccoid isolates (bioMerieux, Inc., St. Louis, Misso.). Identification of the test isolate was carried out by computing and comparing test results from the bioMerieux database. Fatty acid methyl ester profiles were carried out as described previously (Ringelberg *et al.*, 1994).

Molecular characterization. Purified genomic DNA (Johnson, 1981) from liquid-grown cultures were quantified and ~10 ng of DNA was used as the template for PCR amplification. PCR assays were performed in a GeneAmp PCR System 9700 Thermal Cycler (Perkin Elmer Corp., Foster City, Calif.).

(i) 16S rDNA. Universal primers (Bact 11 and 1,492) were used to amplify the 1.4-kb PCR fragment per protocols established by Ruimy *et al.* (1994). Amplicons thus generated were sequenced directly following purification on Qiagen columns (Qiagen, Valencia, Calif.).

(ii) Sequencing. The identity of a given PCR product was verified by sequencing using the dideoxy chain termination method with Sequenase DNA sequencing kit (United States Biochemical Corporation, Cleveland, Ohio) and with an ABI 373A automatic

sequencer as recommended by the manufacturer (Perkin-Elmer Corp.).

(iii) Phylogenetic analysis and sequence alignment. The phylogenetic relationships of organisms covered in this study were determined by comparison of individual 16S rDNA sequences to other already existing sequences in the public database. Evolutionary trees were constructed with the PAUP (Swofford, 1990).

RESULTS AND DISCUSSION

Comparison of conventional bioassay and molecular diagnosis in the detection of microbial population on metal surface

Morphological and biochemical characteristics. Representatives of both Gram-positive and Gram-negative bacterial species that were desiccated on aluminum metal coupons were observed under ESEM and the images are shown in Fig. 1. *B. subtilis* and *D. radiodurans* both are Gram-positives showed prominent morphology where the UV-resistant *D. radiodurans* did not show any damage in its structure. However, the cell components were exuded out in *B. subtilis.* Unlike this, both the Gram-negative forms exhibited remnants rather than their intact cell structure.

Molecular diagnosis of microbes. The universal primer set used in this study generated a 1.5-kb amplicon after PCR amplification. The PCR produced amplicons from both whole bacterial cells without DNA extraction with some exceptions, as well as from purified DNA. In the initial experiments, bacterial suspension was prepared in liquid bacteriological media and was either placed onto glass or metal surfaces. Once the microcosms were dried out on the test surfaces, the nutrient rich media condensed and formed crystalline structures, therefore affected the viability of the cells (data not shown). Similarly, microcosms that were prepared in 0.9% sterile saline solution had similar problems. However, we have avoided this problem by using sterile distilled water as a diluent instead of sterile media or any kinds of buffers.

Recovery of bacterial cells from glass surfaces. Table 1 shows the results of standard plate counts and the sensitivity of PCR technique in the detection of *E. coli* ATCC 25922 from glass surfaces. The PCR method detected *E. coli* from microcosms that contain 9.1×10^1 CFU/ 10 µL PCR mixture. The conventional standard plate counts could not detect any microorganisms from the swabbed materials of artificial biofilms that had even 10^5 CFU/mL. Likewise, whole-cell PCR also did not detect any amplification for the artificial biofilm-microcosms that contain 10^5 CFU/mL. Sterility tests revealed that bacterial populations present in these microcosms were non-viable and non-culturable. However, once DNA was extracted from the glass surface, PCR amplified the target fragment from the microcosm that contain 9.1×10^1 CFU/ 10 µL PCR mixture.

Recovery of bacterial population from metal surfaces. Table 2 shows the results of standard plate counts and the sensitivity of PCR technique in the detection of *B. subtilis*

ATCC 6051 from aluminum metal surfaces. The PCR method detected *B. subtilis* from microcosms that contain 2.64×10^{1} CFU/ 40 µL PCR mixture. The conventional standard plate counts could not detect any microorganisms from the artificial biofilms that had even 10^{5} CFU/mL. However, the liquid culture recovered cells from the microcosms that contain 10^{5} CFU/mL. In addition, PCR detected positive amplification for the microcosms that were not cultivable by conventional techniques.

Influence of dehydration in the removal of artificial microbial biofilms. Table 4 shows the influence of swabbing technique and dehydration on the enumeration of bacterial population in swabbed materials. A 90% reduction in bacterial population was noticed when the microcosm was swabbed within 15 min during which time the artificial biofilm on the metal surface was not yet dehydrated. This may be due to the adherence of bacterial population onto the cotton swabs as well as non-removal of bacterial population from the metal surface by this technique. Further incubation of the artificial biofilm for 24 h at room temperature removed all moisture in the microcosm and no bacterial population was retrieved. Damage to the bacterial cells might be the crucial factor for non-retrieval of any bacterial population. Epifluorescence microscopy revealed patches of bacterial cells adhered onto metal surfaces and supported our claim that the swabbing technique did not remove all bacterial population from the metal surfaces (Fig. 2a). From this experiment, we could conclude that the swabbing technique used here is not removing all bacterial population from metal surfaces and that the cotton swabs used did not release the swabbed biological materials into the solution. This clearly supported our view that the swabbing technique used in this study should be modified to remove all bacterial population that adhered onto the metal surfaces. Fig. 2 further reiterated the fact that the healthy cells showed distinct smooth morphology (Fig. 2b) than the desiccated cells (Fig. 2c and 2d). A spiny like structure was noticed in the desiccated cells (Fig. 2d) which might protect the cells from dehydration thus helping the microbes to survive under extreme conditions. Because of this fact, cells might be difficult to culture using conventional microbiological assays. The enzymes used to break the cell wall thus release the DNA into the solution is also prohibited because of these thick spiny layers (Fig. 2d).

Electron microscopic studies of the dehydrated bacterial population. Whole-cell PCR comprises the introduction of microorganisms as a whole cell for PCR amplification without breaking the cell wall to expose the cellular components such as DNAs or RNAs. Low yield in the whole cell PCR in the dehydrated swabbed samples was disappointing because the PCR procedure is considered highly sensitive and reported to require very low bacterial numbers for positive amplification of the targeted DNA. In order to know the reason behind the inhibition of PCR amplification, samples that were swabbed were subjected to electron microscopy. The swabbed samples were fixed in conventional fixatives before viewing with an electron microscope. The healthy *E. coli* prepared as described in "microcosms A" (Fig. 3a) and dehydrated *E. coli* cells as shown in "microcosms B" from aluminum metal surfaces (Fig. 3b) were scanned for its morphological characteristics. These electron micrographs clearly revealed that physical

damages occurred in bacteria due to dehydration (Fig. 3b). Transmission electron microscopic studies further substantiated that the bacterial cell wall was rigid and wavy in the *E. coli* samples that were dehydrated (Fig. 3d) where as bacterial cell wall structures were normal for cells derived from actively growing cultures (Fig. 3c). In addition to the cell wall damage, cellular materials were displaced towards the cell wall in the dehydrated samples.

Limitations of whole-cell PCR. Although amplification of 16S rDNA was noticed in healthy *E. coli* samples at low concentration by whole-cell PCR, no amplification was observed in dehydrated *E. coli* even though 10^5 cells were presumably present in a given microcosm. This is due to the above said physiological changes such as dehydration, as well as the non-removal of all bacterial population by the technique used to remove bacterial cells from the metal surfaces. Hence, a simple procedure was carried out to break down bacterial cell wall and purified nucleic acid components were released into the PCR reaction mixture. The efficacy of the DNA extraction procedure was evaluated in various bacterial population by comparing with conventional microbiological assays. We have coated various concentrations of 11 different bacterial populations on aluminum metal surface and evaluated the efficacy of the PCR technique in the detection of viable, non-viable and non-cultivable microorganisms (Table 3). These microbes included 6 Gram-positives and 5 Gram-negative bacteria. Among 22 combinations tested, 7 microcosms (35%) showed viability by conventional agar assay. The simple sterility assay exhibited growth in 12 microcosms where as whole cell PCR technique amplified the targeted amplicon in 15 combinations. However once DNA was extracted 91% (20 out of 22 combinations) of the samples showed positive amplification. The absence of amplification from non-cultivable microcosms (2 out of 22 tested) by PCR suggests that bacterial population present in these microcosms might have gone through some morphological and physiological changes. Such rigid structures would have been resistant to enzyme treatments which break open the cell wall and release DNA into the liquid.

Microbial characterization of Spacecraft Assembly Facility

Microbial examination of witness plates. About 20 to 25 replicates of 5 different types of witness plates were removed from SAF and individually placed into 50-mL screw-capped sterile centrifuge tubes. Although we deployed these witness plates on various dates, we have retrieved the samples on the same day. The exposure time varies from 232 days to 278 days. The distribution of particulate materials and fibers collected on various witness plates during the present study showed a definite pattern. In general, particles of the size 5 to 100 μm were collected on all types of witness plates. A clear pattern of particle distribution was noticed in the spacecraft quality paint coated witness plates. The smaller size particles were more abundant in the paint coated witness plates and the profusion decreased with increased particles size. In contrast, pure stainless steel witness plates accumulated more mid-range size (26-100 μm) particles and the abundance of particles decreased when the particles sizes decreased. In terms of fibers, the NS43G

paint coated surface attracted more fibers followed by stainless steel, S-13GP/LO-1, Z307 and 463-3-8 paint coated surfaces.

Heat-resistant and vegetative microbial population enumerated from various witness plates that were exposed at JPL-SAF are depicted (Fig. 4). In general, the paint-coated witness plates attracted more microbial population than the pure stainless steel plates. Such microbial abundance is well correlated to an abundance of particulate materials trapped on the witness plates. In other words, NS43G-coated witness plates had trapped more particles and the number of microorganisms isolated was also higher when compared to other witness plates. However, the number of fibers on various witness plates showed no relationship with the microbial counts. In general, both heat-resistant and vegetative microbial contamination transferred through particulate materials is not heavy and it is minimal in terms of microbial load. In addition to the bacterial population, spatial distribution of yeast and fungal population were noticed in these witness plates and were insignificant. Characteristics of microbes other than bacteria were not carried out during this study.

A spacecraft building facility for a life detection mission is often guarded by stringent quality control measures. Because of controlled air circulation, desiccation, moderately high temperature, and low-nutrient conditions, the environment of JPL-SAF can be considered as extreme environment and microbes might find difficult to thrive under such conditions. Evidently, many bacterial strains isolated from the JPL-SAF exhibited intense growth at 60°C (44%), and 10% NaCl (50%). This substantiated the fact the JPL-SAF is an extreme environment. A majority of these thermotolerant and halotolerant isolates were identified as the *Bacillus* species. Our results confirmed that the JPL-SAF High Bay-1 exhibited lower microbial burden and their taxonomical characterization revealed a similar microbial profile as that of the Viking mission (Puleo et al. 1975). In other words, the Precursor spacecraft that used to verify spacecraft level flight article assembly and test operating procedures at the launching site had high incidence of the members of the *Bacillaceae* family. The witness plates employed in this study mimics the Precursor spacecraft in their microbial profile rather than the Viking 1 and 2 spacecraft.

Phenotypic characterization. Representatives of rod-shaped and coccoid-shaped bacterial cultures were tested for their physiological characteristics features using commercially available test strips. Based on the Biomerieux database, only 9 rods and 1 coccoid-shaped bacteria were identified to its species level. All rod-shaped bacteria were presumptively identified as *Bacillus* sp. and fell into two groups: *B. licheniformis* and *B. pumilus.* The coccoid-shaped isolates were identified by phenotypes as members of the genera *Micrococcus* and *Staphylococcus.*

Fatty acid analysis. The FAME compositions of various strains isolated showed that the terminally branched saturated FAME (58.3 to 99.2%) peaks were the major lipids when compared to straight chain saturated FAME (2.9 to 41.7%) and monounsaturated

FAME (0.8 to 30.2%) lipid classes. Among coccoid-shaped groups, members of *Micrococcus* genus (FO-017a, FO-084a) had shown unique FAME patterns (80% are 15:0) when compared to others. Likewise, strain *P. citreus* FO-074a (19% monounsaturated) and *S. capitis* FO-036a (41% straight chain saturated) also showed different patterns in their FAME composition. Among various groups of *Bacillus, B. licheniformis*-group yielded significant amount of 15:0 anteiso (33 to 41%), 15:0 iso (20 to 34%) and 17:0 anteiso (11 to 16%). One unidentified *Bacillus* sp. FO-092 also fell into this FAME category. *B. pumilus*-group (FO-033, FO-036b, FO-038) generated significantly more 15:0 iso (45 to 49%) than 15:0 anteiso (25%). The strains FO-003 and FO-029a were higher in C15:0 anteiso (40 to 41%) than C15:0 iso (13 to 20%). Unlike those seen in any other group, *B. cereus*-group (FO-011, FO-080) produced significant amounts of monosaturates (20 to 30%) among their total FAME composition.

16S rDNA sequence analysis. There were 3 different clades observed among coccoid-shaped bacteria (Fig. 5). They were the members of the genera *Micrococcus, Staphylococcus,* and *Planococcus.* The phylogenetic tree based on the 16S rDNA analysis indicates that the *Micrococcus* species forms a group independent of the others. *Staphylococcus* and *Planococcus* species form distinct independent groups and these 2 groups were clustered in the main stalk of *Bacillus* species. The strain FO-036a showed 99.8% similarities in its 16S rDNA sequence to both *S. epidermidis* and *S. capitis.* However, the strain FO-074a, had low similarities to the sequences available in the public database. The genus *Planococcus* is the closest relative to the strain FO-074a and shows 96.9% similarities to *P. citreus.*

Variation in 16S rDNA sequences among rod-shaped strains revealed 5 groups (Fig. 5). These are related to *B. cereus*-group (2 strains), *B. licheniformis* (12 strains), *B. pumilus* (3 strains), *B. subtilis* (1 strain) and *B. circulans* (1 strain) group. This grouping was drawn on the basis of their proximity to respective type strains. A group of 12 strains that are related to *B. licheniformis*, while somewhat heterogeneous in 16S rDNA sequence (97.2 to 99.8% similarities) appeared to form a cluster. Both *B. licheniformis* and *B. pumilus* groups occupy the same stem in the phylogenetic tree, in which the strain FO-029a that showed higher similarities to *B. subtilis* (99%) also grouped together. The strain FO-011 that was identified as *B. cereus* exhibited 99.1% similarities to both *B. cereus* and *B. thuringiensis.* Likewise, the other member of this cluster, strain FO-080, exhibiting mucoid morphology, did cluster with *B. mycoides* (99.2%) however this strain showed 98.9% similarities to both *B. cereus* and *B. thuringiensis.* The lone member of the fifth group, the strain FO-092, with high similarities to *B. circulans,* formed a cluster with *B. benzeovorans* (98.1%) and *B. circulans* (98.7%). Although the strain FO-092 exhibited high similarities to *B. firmus* (98.3%), and *B. megaterium* (97.6%), these species bifurcated from FO-092-cluster at the stem of the tree.

DNA-DNA hybridization. Among the 19 *Bacillus* strains tested, the DNA reassociation percentage range was 77 to 100% for 10 *B. licheniformis* strains. The strain

FO-035b that show higher 16S rDNA sequence similarities (99%) to *B. licheniformis* did not exhibit higher DNA reassociation values (38%) between them. One of the two strains identified as *B. cereus*-group (FO-080) showed 77% similarities to *B. mycoides* whereas the non-mucoid strain FO-011 exhibited 53 to 61% similarities to the members of *B. cereus*-group. Three strains showed closest match to *B. pumilus* (65 to 85%). The strain FO-029a that shows 99% 16S rDNA similarities to *B. popilliae* did exhibit 77% DNA hybridization similarities with *B. subtilis* type strain. Similarly, the other member of the *B. subtilis* group (FO-032) that clustered with *B. licheniformis* based on 16S rDNA phylogeny (99%), showed higher DNA dissociation value (88%). One morphologically novel strain (FO-092) that produces extremely big spores (data not shown) showed no relationship with any of the *Bacillus* species tested. Furthermore, the FO-092 strain that exhibited 98.7% 16S rDNA sequence similarities with *B. circulans* showed only a 21% DNA reassociation value.

Among the five coccoid strains examined, two strains (FO-017a and FO-084a) showed higher DNA reassociation values among themselves (>80%) and with *Micrococcus luteus* (~81%). The strain FO-074a exhibited 81% value with *Planococcus citreus* IFO 15849. The strain FO-036a revealed 89% and 70% hybridization values with *S. capitis* subspecies *capitis* ATCC 27840 and *S. capitis* subspecies *ureolyticus* ATCC 49326, respectively.

Comparative phylogeny of 16S rDNA in the family *Bacillaceae* was recently documented (Achouak et al., 1999). The sequence analysis has demonstrated that the genus *Bacillus* consists of at least five monophyletic clusters (Ash et al., 1993-94). *Bacillus* species that produce acid from a variety of sugars including glucose were classified under rRNA group 1. Most of these species were able to grow at least weakly in the absence of oxygen. Spores of these species were ellipsoidal and did not swell the mother cell. These species are considered the "subtilis group" because of their otherwise similar physiological properties (Priest, 1993). All Gram-positive rods isolated from JPL-SAF fell into the rRNA group 1. The members of the "subtilis group" (*B. cereus, B. licheniformis, B. pumilus,* and *B. subtilis*) are prevalent in soils, dusts, marine and freshwater habitats. This microbial profile of JPL-SAF High Bay 1 resembles the Kennedy Space Center SAF, Florida, where the Precursor spacecraft of the Viking mission were kept.

Because 16S rDNA sequence analysis has proven inadequate for the differentiation of *Bacillus* species, DNA-DNA hybridization was used to verify our assertion that any of these strains described here deserves species status. The conventional biochemical tests, FAME analysis and 16S rDNA sequence results did not firmly define the species status of the given strain during this study. Only 4 out of 19 *Bacillus* strains and one of the five coccoid-shaped shaped strains were identified as the same species by all four methodologies. It is clear that the biochemical test profile did not differentiate the Gram-positive bacteria at their species level. This might be due to the fact that the microbes isolated from SAF would have to shed some of their genetic characters to adapt

themselves to the extreme environmental conditions thereby changing their phenotypes. The results of FAME and 16S rDNA profiles (85%), and 16S rDNA and DNA-DNA hybridization profiles (75%) were in accordance in identifying them as the same species. DNA hybridization studies revealed existence of 4 established *Bacillus* species among 19 strains tested and further supported to describe 3 new *Bacillus* species. It is interesting to note that the strain FO-092 had a clear match with *B. circulans* by phenotypic data, FAME profiles, and 16S rDNA sequence similarities (98.7%). However, DNA hybridization study (21% with *B. circulans*) clearly indicates that the strain FO-092 should be regarded as distinct.

The work at hand represents a comprehensive effort to impose a logical phylogenetic framework on the growing collection of SAF isolates. While it appears likely that additional isolates representing novel species will be discovered in the future, the species considered here already indicate that JPL-SAF contains formerly unappreciated microbial diversity. This study illustrates the utility of molecular and chemotaxonomical approaches for determining phylogenetic relationships in SAF. It is possible to build upon the taxonomic foundations established by the polyphasic approach to design gene probes for the rapid and efficient screening of microbes to ensure the microbial quality of the facility.

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FIGURE LEGENDS

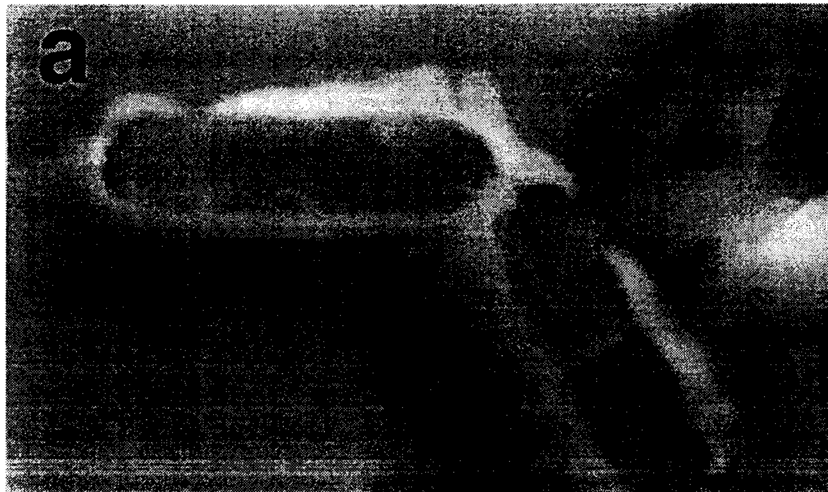
Fig. 1. Environmental scanning electron micrographs of various microorganisms employed.

Fig. 2. Microscopic examinations of desiccated and healthy *D. radiodurans* on aluminum surfaces. (a) Epifluorescence image after BacLight (live-dead) staining; (b-d) ESEM images; (b) healthy cells; (c, d) desiccated cells

Fig. 3. Electron micrographs of healthy and desiccated *E. coli* cells. a and b, scanning electron microscopy; c and d, transmission electron microscopy.

Fig. 4. Microbial characteristics of various witness plates deployed at SAF.

Fig. 5. Phylogenetic tree of the microbial strains isolated from the JPL-SAF based on 16S rDNA sequence comparison by maximum likelihood analysis.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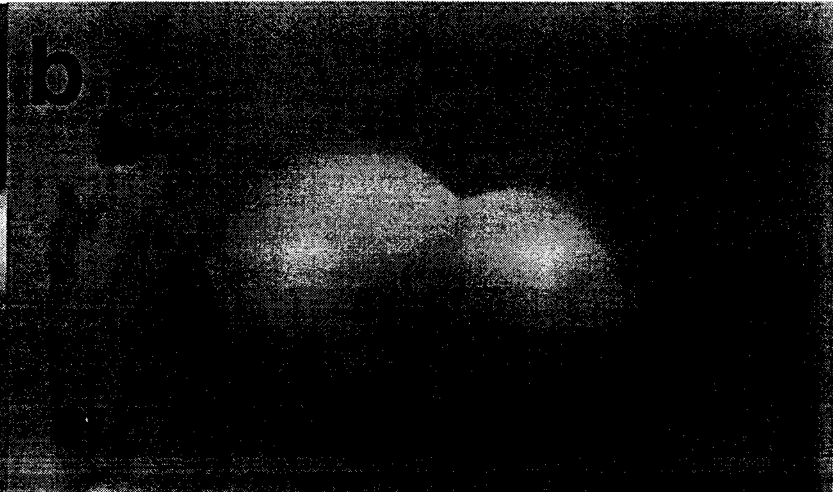
1 µm
10.0 kV 20000x SE 17.6 57
Bacillus subtilis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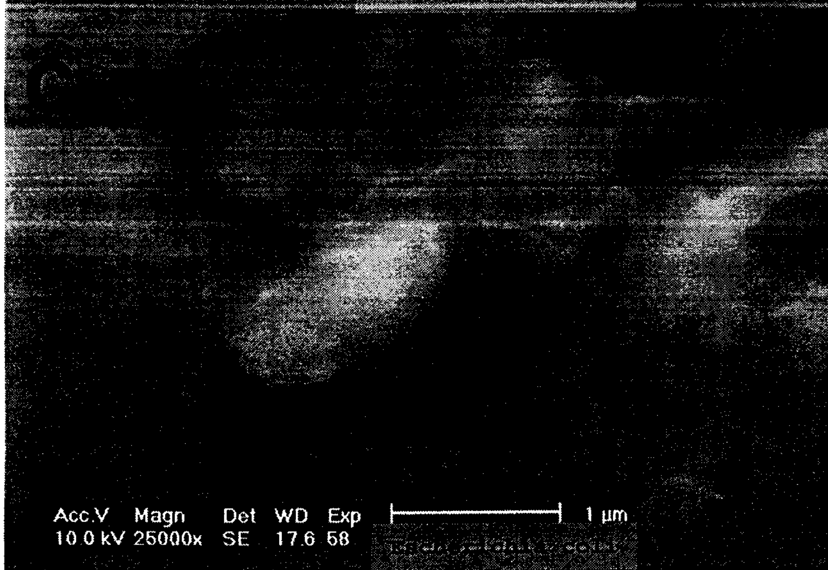
Deinococcus radiodurans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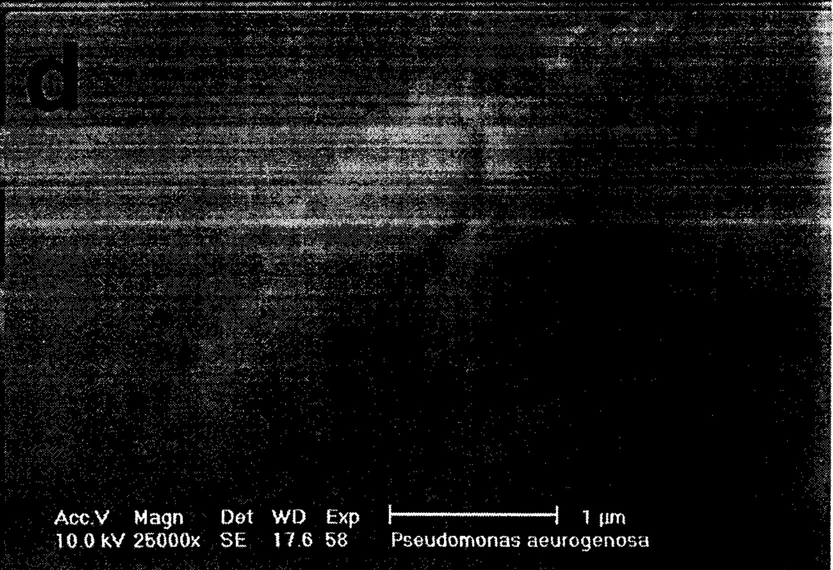
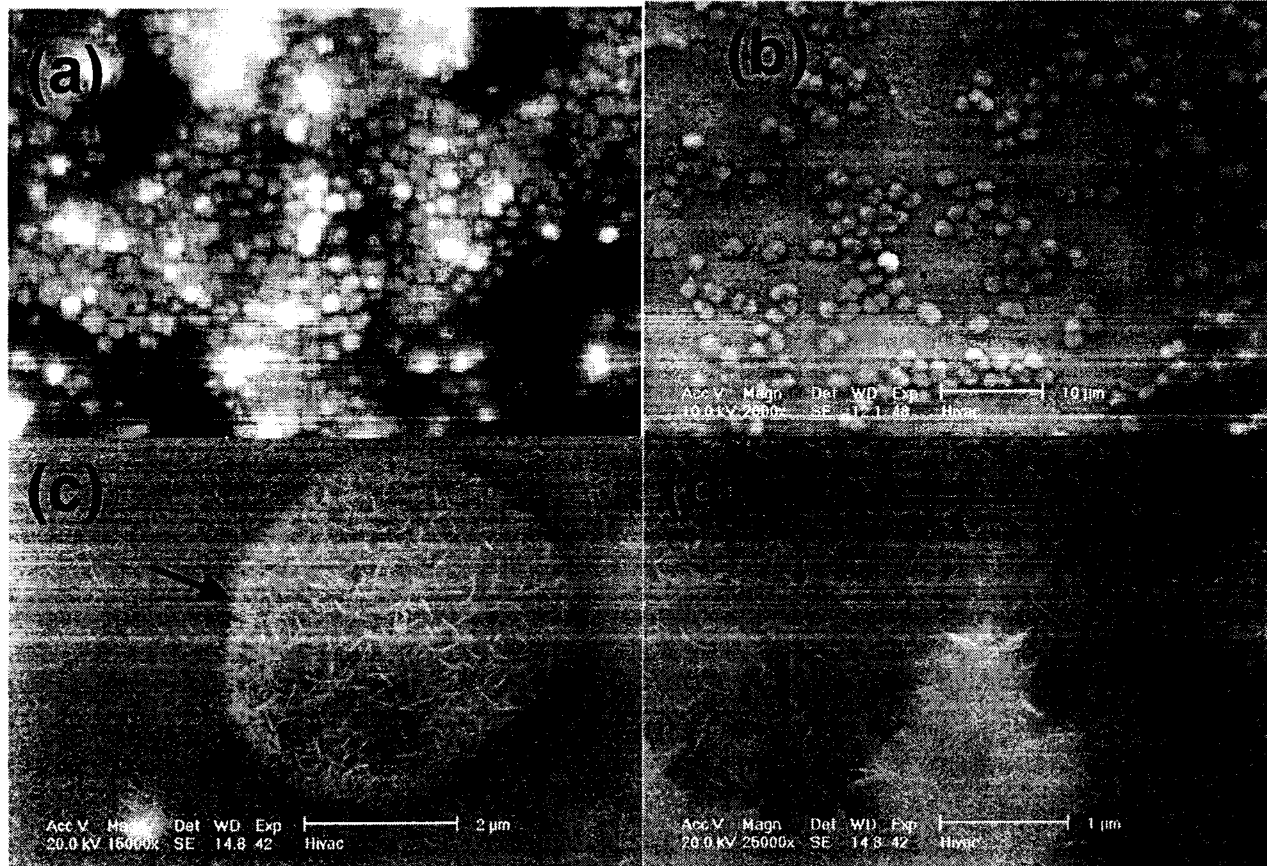

d
Acc.V Magn Det WD Exp
1 µm
10.0 kV 25000x SE 17.6 58
Pseudomonas aeurogenosa


(a)
(b)
Acc.V Magn Det WD Exp
10 µm
(c)
Acc.V Det WD Exp
20.0 kV 16000x SE 14.8 42 Hivac
2 µm
Acc.V Magn Det WD Exp
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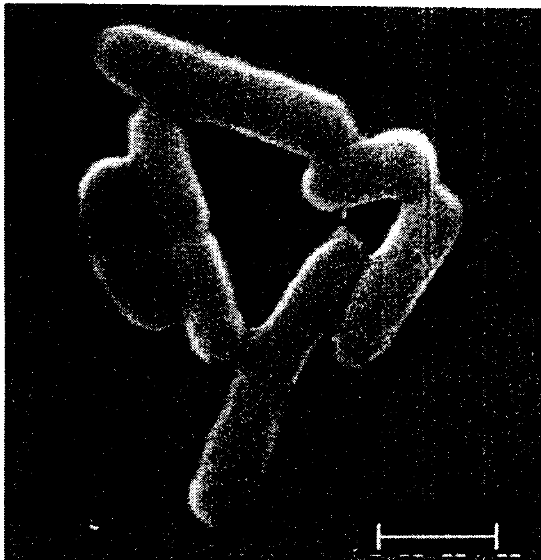
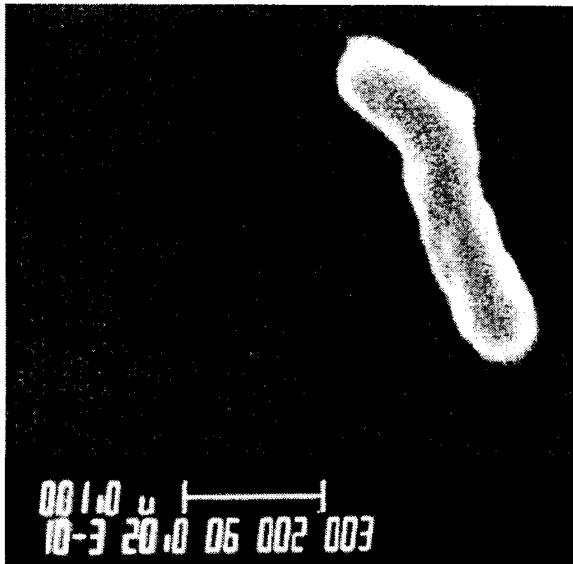
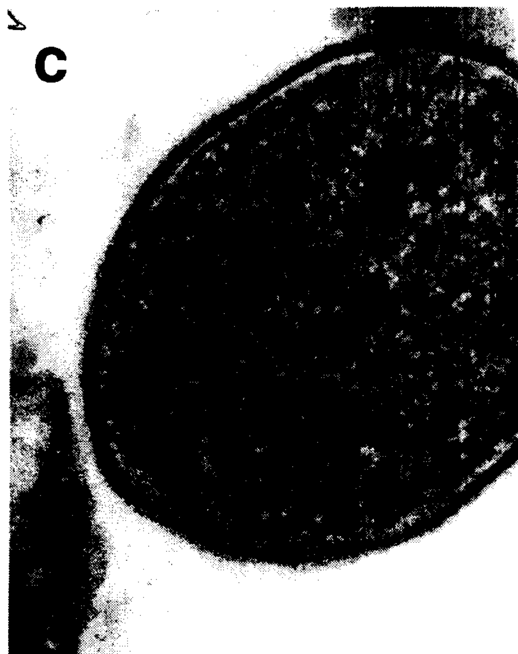
1 µm



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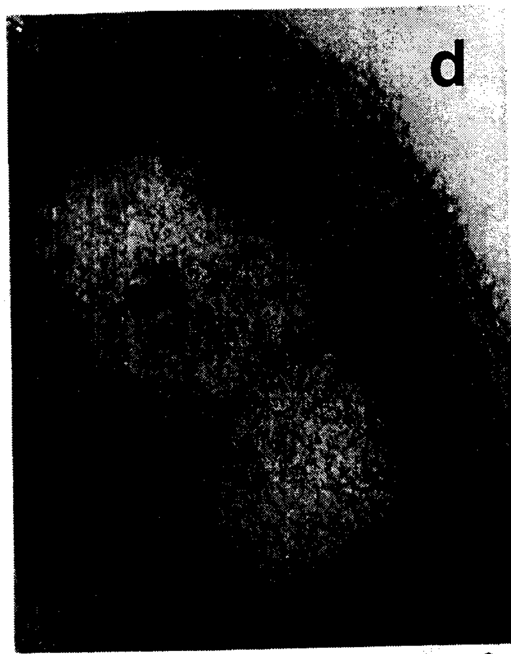
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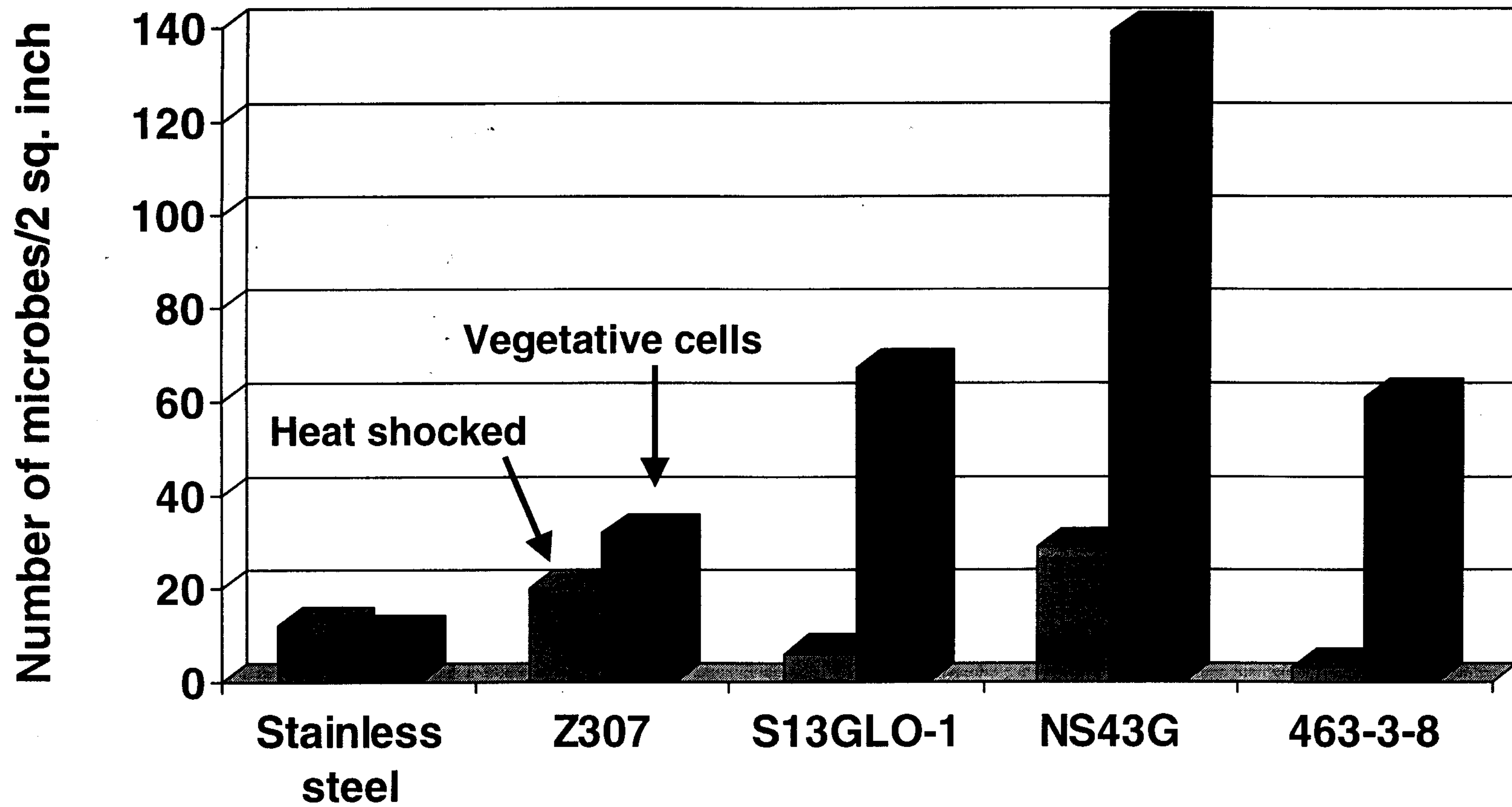
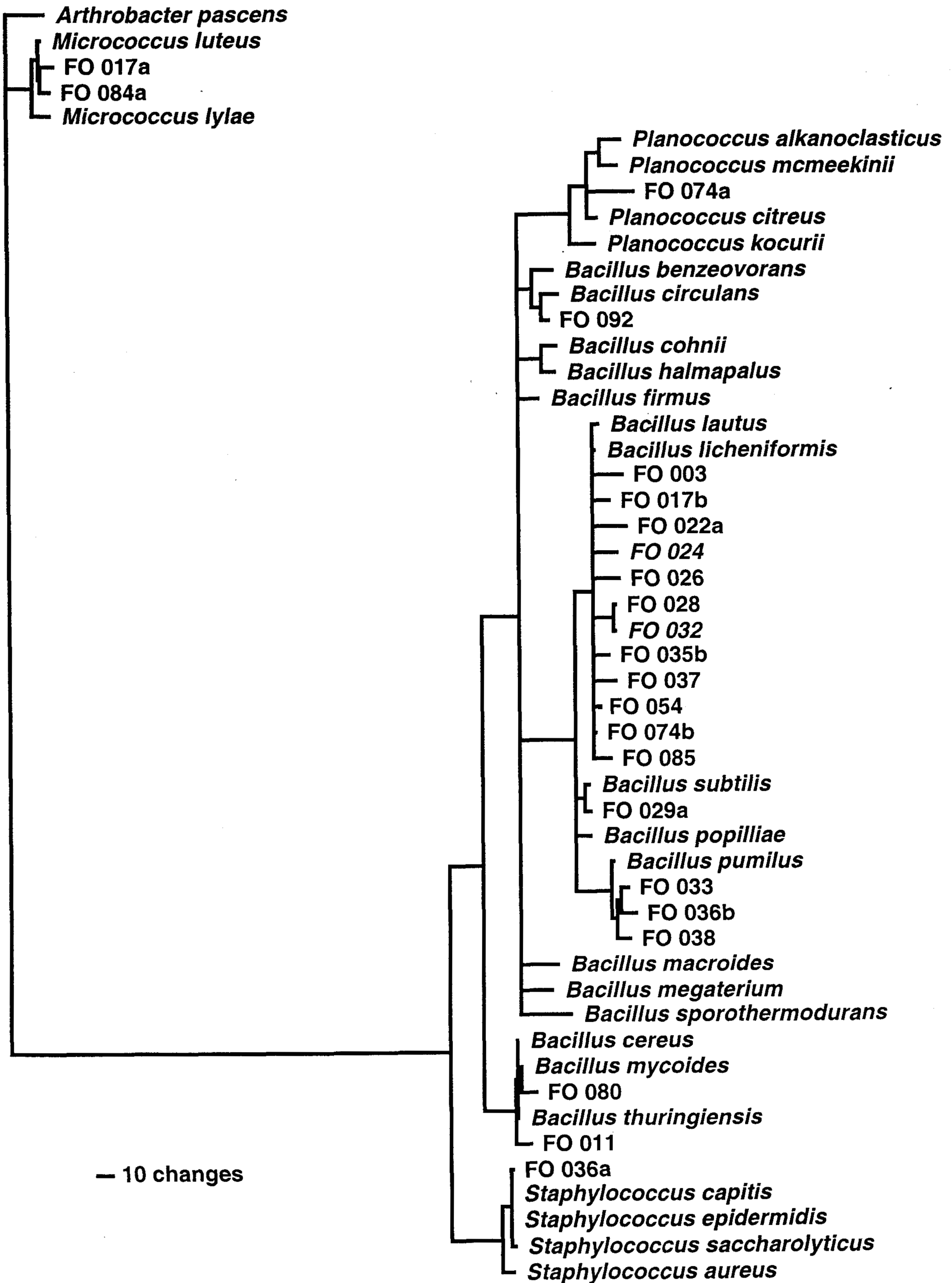

Number of microbes/2 sq. inch
140
120
100
80
60
40
20
0
Vegetative cells
Heat shocked
Stainless steel
Z307
S13GLO-1
NS43G
463-3-8



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FO 084a
Micrococcus lylae
Planococcus alkanoclasticus
Planococcus mcmeekinii
FO 074a
Planococcus citreus
Planococcus kocurii
Bacillus benzeovorans
Bacillus circulans
FO 092
Bacillus cohnii
Bacillus halmapalus
Bacillus firmus
Bacillus lautus
Bacillus licheniformis
FO 003
FO 017b
FO 022a
FO 024
FO 026
FO 028
FO 032
FO 035b
FO 037
FO 054
FO 074b
FO 085
Bacillus subtilis
FO 029a
Bacillus popilliae
Bacillus pumilus
FO 033
FO 036b
FO 038
Bacillus macroides
Bacillus megaterium
Bacillus sporothermodurans
Bacillus cereus
Bacillus mycoides
FO 080
Bacillus thuringiensis
FO 011
10 changes
FO 036a
Staphylococcus capitis
Staphylococcus epidermidis
Staphylococcus saccharolyticus
Staphylococcus aureus