The Search for Thin Bacteria

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Abstract: Whole bacterial cells can be reconstructed in 3-D to ~ 5 nm resolution using cryoelectron tomography, provided the cells' thickness is narrower than 350 nm. Towards the goal of reconstructing interesting bacteria, thin specimens were sought. Microbial sand mats or sediment were placed over fine (0.2 μm) membrane filters overlaying two types of media: a dilute yet complex Tryptone and Yeast Extract media (LTY) and a complex Multipurpose Phototroph Isolation (MPI) media. After several hours, sediment was removed and plates were incubated aerobically in LTY or anaerobically in MPI. Any colonies found were sequentially restreaked to the third iteration. The most common cell diameter obtained was 0.4 μm, with some cultures narrower, but most were wider, probably due to contamination. Firmicutes, Gamma- and Epsilonproteobacteria were definitively obtained, though all cell shapes were present in the collection, including spirochetes. Liquid cultures were also passed through coarse and fine syringe filters, and mixed cultures of ~ 0.4 μm bacteria were obtained in this way. Thin spore formers, truly phototrophic thin bacteria and thin archaea were not obtained.

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Introduction

The thinner the cells, the better the cryoelectron tomographic reconstruction. Cryoelectron tomography is an imaging strategy wherein projections of a sequentially-tilted specimen are acquired in an electron microscope, and the assemblage of images is in turn used to reconstruct the original three-dimensional object. Electrons cannot penetrate specimens thicker than 1 μm, and ideally, the specimen should be thinner than the mean free inelastic path of an electron, which in most cases is 350 nm. Like shooting a bullet into a forest, the thinner the copse, the more likely the bullet will travel through. If the cell is thin, then the cell can be reconstructed to ~ 5 nm resolution. The author is a structural biologist and wishes to obtain good reconstructions of interesting bacteria, and so, the goal is to isolate thin bacteria from the environment.

Luckily, there are thin bacteria. Because bacteria are limited by diffusion for the acquisition of food, and cannot acquire more by swimming faster (like a baleen whale gathering krill), there is a selective advantage in being narrow[1]. The higher ratio of surface area to volume of thin bacteria means they can transport relatively more food into their interior volume than wide ones.

Thin bacteria can be collected by taking advantage of many bacteria's chemotaxis and motility[2]. By setting a coarse or fine (0.45 or 0.2 μm) membrane filter between an environmental sample and a media containing dilute quantities of a high quality media, chemotactic cells can sense the gradient, swim towards the filter, and if they are narrow, pass through. Not all bacteria are chemotactic or motile, so another strategy is to gently push a liquid sample through a syringe filter into another, connected, media-filled syringe. Both methods were used to obtain bacteria of various diameters.

Methods

Samples were taken from sand mats, deep sediment and soil. Sand mats were used from the Trunk River, the Sippewissett Marsh and the School St. Marsh. Sediment came from the Cedar swamp. Soil came from behind Swope and also near the Virgin Mary statue of St. Phillip's church. The liquid samples were two, green, turbid and several-week-old solutions that overlaid sand mats contained in 50 ml Falcon tubes. One was from the Sippewissett and the other from the Trunk River.

The first method was to place a moist, solid sample over a nylon Millipore, 0.2 μm membrane filter (type GNWP) that overlaid one of two kinds of media. The first media called LTY contained 0.1 grams per Liter of Tryptone and Yeast Extract in either a freshwater or saltwater base (FW LTY or SW LTY, resp.).[3] Using higher concentrations of the complex ingredients is not advisable because the nutrients may diffuse into the moist sample rather than entice thin bacteria through the filter. The second media was the Multipurpose Phototrophic Isolation media (MPI). It contained 5 mM Ammonium chloride, 1.5 mM Potassium phosphate, 10 mM MOPS buffer pH 7.2, Trace elements, Multivitamins, 250 mM Sodium Sulfate, 10 mM Sodium Thiosulfate, 13 mM Sodium Acetate, 70 mM Bicarbonate and 0.075 g Yeast Extract per liter (see Appendix). The ammonium may serve as a nitrogen source; the sulfate and thiosulfate as sulfur sources or as electron acceptors (for sulfate reducers); the acetate may serve as either a carbon source or an electron donor; and the bicarbonate as carbon source (See Appendix).

To entice anaerobic phototrophs through a filter, MPI plates were incubated in an anaerobic jar over a light bulb (Figure 1A). As a control, the same samples were also placed

over aerobic LTY plates. To obtain thin archaea, MPI plates were created that contained 100 μg/ml of the antibiotics rifamycin, ampicillin and tetracyclin. To obtain thin spore formers, the soil or sediment was boiled for 15 minutes, then placed over the filter and allowed to rest for 1 day. After the incubation period, the filter was removed and the plates turned upside down. For all attempts, if a colony was found within the filter's former location on the plate, it was restreaked up to 3 times to isolate a pure culture.

The second method was to load a liquid sample into a 5 ml syringe and push the contents through a coarse filter into an attached 1 ml syringe containing SW LTY media. The accepting syringe was attached to the filter outlet with rubber hose (6 mm exterior diameter and a 3 mm internal diameter). See Figure 1B. The two syringes were pressed back and forth three times, and then the apparatus was allowed to sit for around three days.

Results

Table 1 shows which colonies were found, restreaked and grown to completion. Twenty-four moist samples were placed over fine filters over media. Thirty-five resulting colonies were restreaked once; twenty-three were restreaked twice and eleven were restreaked thrice. Two grew up in liquid culture. Eleven cultures were successfully sequenced. Twenty-five were imaged and their diameter estimated. The supplementary CD has all figures and movies taken of the various thin bacteria. All variety of cell shapes were obtained: cocci, rods, vibroid, spirilla and spirochetes. The histogram of cell diameters is shown in Figure 3. The most common diameter was 0.4 μm with ten instances. Six cultures were narrower than 0.4 μm, but nine others were thicker than 0.5 μm. The thicker cells were probably contaminants. Two liquid cultures were passed through a 0.45 μm filter and both produced many cell morphologies in the receiving syringe. One successful filtration was further passed through a 0.2 μm filter, and various and thin (0.4 μm) cell morphologies passed through.

For plates incubated aerobically, Firmicutes and Gammaproteobacteria were isolated (Figure 2). Species like *Bacillus firmus* (0.4 - 0.9 μm diameter) and *Bacillus luciferensis* (1 μm) were obtained, as were *Marinobacter hydrocarbonoclasticus* (0.4 μm) (See Figure 4). Many others were obtained that could not be identified in time. A hindrance occurred when, in doing nested PCR, i.e., using a weak PCR product as template for an additional round, the background bacteria present in the reagents was amplified, namely, *Ralstonia pickettii*. That *R. pickettii* is resistant to heavy metals, and that it is present in MBL's water, should make one pause before drinking MBL's water.

For plates incubated anaerobically, more cultures were identified successfully as either Gamma- or Epsilonproteobacteria. From Trunk river sand mats were isolated ~ *Vibrio diazotrophicus* (0.7 μm) and ~ *Microbulbifer maritimus* (0.4 μm). From Sippewissett Marsh sand mats came ~ *Arcobacter nitrofigilis* (0.3 - 0.4 μm), and from a School St. Marsh sand mat came ~ *Sulfurospirillum arcachonense* (0.4 μm).

For two liquid samples, fast, motile bacteria passed through a 0.45 μm syringe filter successfully. The cells were up to 0.8 μm in diameter. For the Sippewissett sample, the filtrate was passed through another finer, 0.2 μm syringe filter, and bacteria up to 0.4 μm were obtained.

No truly phototrophic thin bacteria or thin archaea were obtained.

The mixed or pure cultures were jabbed into agar tubes and will be restarted eventually.

Discussion

Obtaining thin bacteria from the environment is often successful. Thin phototrophs are not yet within grasp, nor are thin archaea. The techniques used will be used again to pursue the undiscovered. To obtain thin phototrophs, it may be necessary to lay a sample over a coarse filter. It may be that because of their large, interior liqht-harvesting assemblies, no phototroph exists below 0.45 μm. To obtain thin archaea, it may be necessary to sample more inhospitable environments where there is a diversity of archaea, e.g. a hot spring.

References

1. John Breznak, "Being Small. The impact of diffusion and viscosity on microbial life." Lecture presented on June 25, 2007 at the Microbial Diversity course.
2. Canale-Parola, E., Rosenthal, S. L., Kupfer, D. G. Morphological and physiological characteristics of *Spirillum gracile*, sp. n. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **32**: 113-124. (1966)
3. Enrichment media recipe from page 3.21 of the Microbial Diversity Manual 2007

Figures

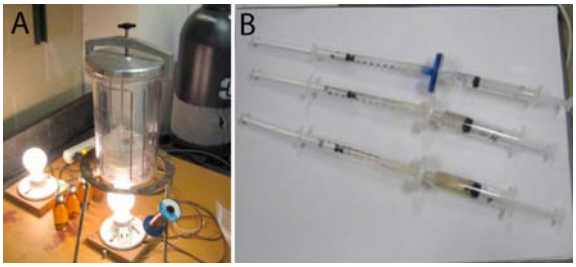

Figure 1. The phototaxis setup and the dual syringe setup
Liquid sample is pressed back and forth across the filter then allowed to incubate.

Figure 2. Phylogenetic tree of successfully sequenced "thin" bacteria
Plates incubated aerobically produced Gammaproteobacteria and Firmicutes, while plates incubated anaerobically produced Gamma- and Epsilonproteobacteria. To conserve space, cultures were renamed. Cultures named GMNP#* are the anaerobic "phototrophs," those named GM#* are aerobic, and those named GMP#* are the aerobic controls of the anaerobic phototrophs. For example, GM2IIIa or GM2IIIb corresponds to Thin2-IIIb in Table 1. GMNP4I corresponds to An-Ph4-I in Table 1.

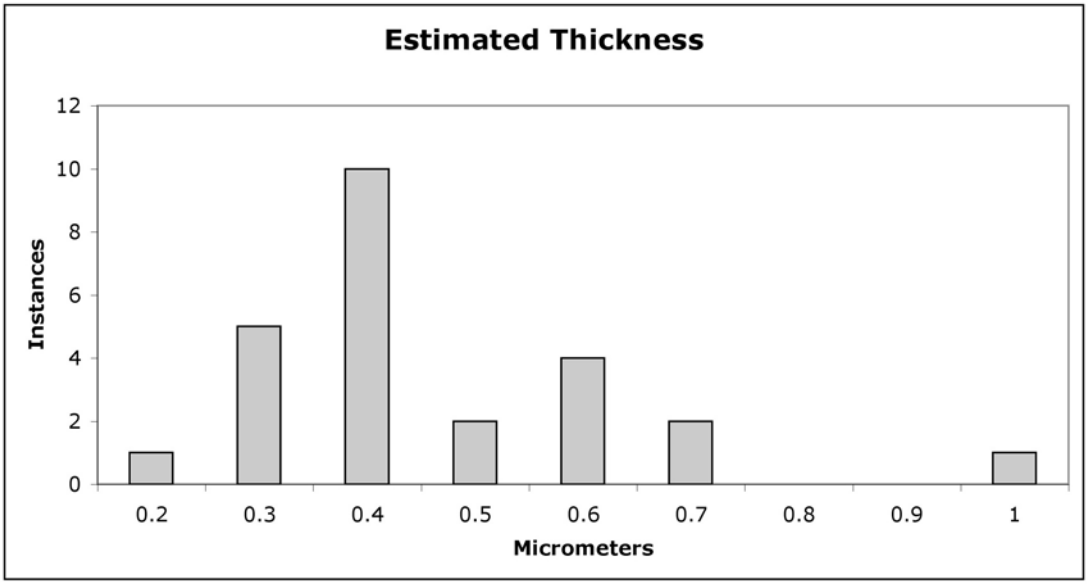

Figure 3. Histogram of Cell Diameters
The most common cell thickness was 0.4 μm

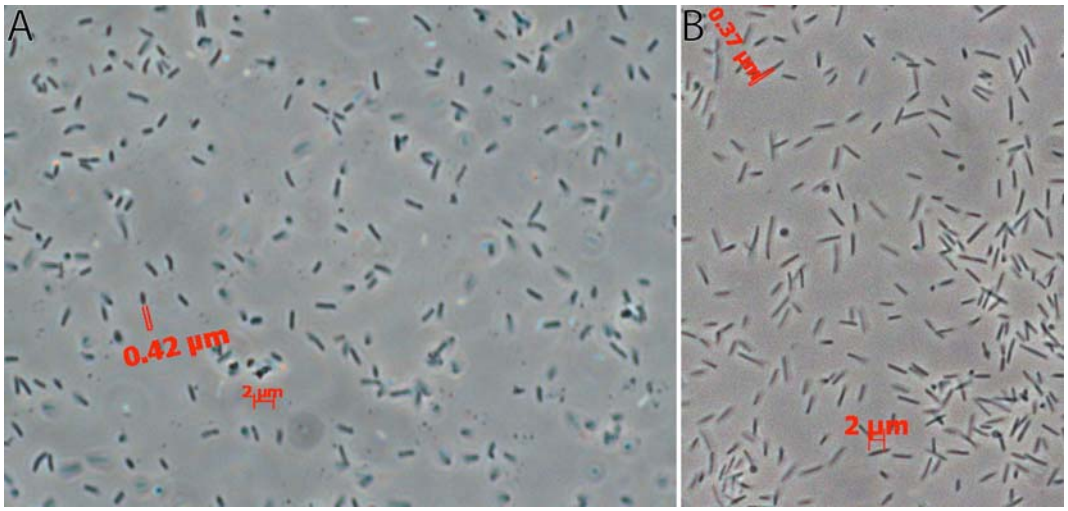

Figure 4. Isolates from the Trunk river
A. When incubated on SW LTY plates aerobically, a *Marinobacter* species (culture Thin 1-I) was isolated. B. The same sample incubated anaerobically on MPI plates produced a *Microbulbifer* species (AnPh1-I).

Table 1
See below.

	A	B	C	D	E	F	G	H	I	J	K
1	Culture	SampleFrom	SamplePrep	Date Started	PlateUsed	PlateTreatment	ColonyMorph	Restreak1	R1_DiffPlateUsed?	R1 Result	Restreak2
2	Thin1-I	OliviaTrunkRiverSandMat		26-Jun-07	SW LTY	Aerobic, on bench		28-Jun-07		spread from s	9-Jul-07
3	Thin1-II	OliviaTrunkRiverSandMat		26-Jun-07	SW LTY	Aerobic, on bench		June 9 & 21		growth from 7	25-Jul-07
4	Thin2-I	SippewissettMarshSandMat		26-Jun-07	SW LTY	Aerobic, on bench	white mound	week3			9-Jul-07
5	Thin2-II	SippewissettMarshSandMat		26-Jun-07	SW LTY	Aerobic, on bench		June 9, 21 & July 9		no growth	
6	Thin2-IIIb	SippewissettMarshSandMat		26-Jun-07	SW LTY	Aerobic, on bench	white disk	week3			Jul 9 & 13
7	Thin2-IIIs	SippewissettMarshSandMat		26-Jun-07	SW LTY	Aerobic, on bench	faint blob	week3			9-Jul-07
8	Thin3-I	MyTrunkRiverSandMat		26-Jun-07	SW LTY	Aerobic, on bench		week3			
9	AnPh1-I	OliviaTrunkRiverSandMat		11-Jul-07	MPI	Anaerobic, Light		16-Jul-07			20-Jul-07
10	AnPh1-II	OliviaTrunkRiverSandMat		11-Jul-07	MPI	Anaerobic, Light		16-Jul-07			20-Jul-07
11	AnPh1-III	OliviaTrunkRiverSandMat		11-Jul-07	MPI	Anaerobic, Light		20-Jul-07			26-Jul-07
12	AnPh2-I	OliviaSippewissettSandMat		11-Jul-07	MPI	Anaerobic, Light		16-Jul-07			20-Jul-07
13	AnPh3-I	JarrodSchoolStSandMat		11-Jul-07	MPI	Anaerobic, Light		20-Jul-07			26-Jul-07
14	AnPh3-II	JarrodSchoolStSandMat		11-Jul-07	MPI	Anaerobic, Light		20-Jul-07			26-Jul-07
15	An-Ph4-I	GeertSippewissettSandMat		11-Jul-07	MPI	Anaerobic, Light		16-Jul-07			20-Jul-07
16	AnPh4-II	GeertSippewissettSandMat		11-Jul-07	MPI	Anaerobic, Light		16-Jul-07			20-Jul-07
17	AnPh4-III	GeertSippewissettSandMat		11-Jul-07	MPI	Anaerobic, Light		16-Jul-07			20-Jul-07
18	AnPh4-IV	GeertSippewissettSandMat		11-Jul-07	MPI	Anaerobic, Light		20-Jul-07			
19	AnPh4-V	GeertSippewissettSandMat		11-Jul-07	MPI	Anaerobic, Light		20-Jul-07			26-Jul-07
20	AirPh1-I	OliviaTrunkRiverSandMat		11-Jul-07	SW LTY	Aerobic, on bench		16-Jul-07			
21	AirPh2-II	OliviaSippewissettSandMat		11-Jul-07	SW LTY	Aerobic, on bench	tiny mound	16-Jul-07		wet droplet, turned red, depression	21-Jul-07
22	AirPh2-III	OliviaSippewissettSandMat		11-Jul-07	SW LTY	Aerobic, on bench					23-Jul-07
23	AirPh4-I	GeertSippewissettSandMat		11-Jul-07	SW LTY	Aerobic, on bench	white spreader	16-Jul-07		many col's	21-Jul-07
24	AirPh4-II	GeertSippewissettSandMat		11-Jul-07	SW LTY	Aerobic, on bench	white mound	16-Jul-07		lots of col's	21-Jul-07
25	AirPh4-III	GeertSippewissettSandMat		11-Jul-07	SW LTY	Aerobic, on bench	clear blob that spreads	16-Jul-07		lots of col's	
26	AirPh4-IV	GeertSippewissettSandMat		11-Jul-07	SW LTY	Aerobic, on bench	orange mound	24-Jul-07			
27	Thin5-I	CedarSwamp		11-Jul-07	SW LTY	Aerobic, on bench	tiny white mound	16-Jul-07			
28	Thin5-II	CedarSwamp		11-Jul-07	SW LTY	Aerobic, on bench	spreading white	16-Jul-07			7/20-7/21/2007
29	Thin5-III	CedarSwamp		11-Jul-07	SW LTY	Aerobic, on bench	faint white blob	16-Jul-07			
30	Thin5-IV	CedarSwamp		11-Jul-07	SW LTY	Aerobic, on bench		21-Jul-07			
31	Thin6-I	CedarSwamp		11-Jul-07	SW LTY	Aerobic, on bench	white mound	21-Jul-07			
32	Thin7-I	CedarSwamp		11-Jul-07	FW LTY	Aerobic, on bench		21-Jul-07			
33	AirSpKim	KimSoilUnderTree	Boiled 15'	12-Jul-07	FW LTY	Aerobic, on bench	white disk under agar	17-Jul-07	FW LTY & Nut.Agar	lots of col's	22-Jul-07
34	AnSprMary	MySoilNearMaryStatue	Boiled 15'	12-Jul-07	FW LTY	Anaerobic	wet droplets	7/18 & 7/21/2007	FW LTY & Nut.Agar		
35	AnSprKim	KimSoilUnderTree	Boiled 15'					18-Jul			
36		BlackSipMarshSandMat	Boiled 15'	12-Jul-07	SW LTY	Anaerobic					
37		MyTrunkRiverSandMat	Boiled 15'	12-Jul-07	SW LTY	Anaerobic					
38	0.45LiqFilt	MyOldTrunkRiverLiqTube	SyringeFilter 5days	20-Jul-07	SW LTY liquid	5 days in tube		25-Jul-07		lots of tiny col	26-Jul-07
39	0.45LiqFilt	MyOldSippewissettLiqTube	SyringeFilter 5days	20-Jul-07	SW LTY liquid	5 days in tube		25-Jul-07		lots of big col'	26-Jul-07
40	Archaea1	TrunkRiverSediment		12-Jul-07	Antibiotic MPI	Anaerobic					
41	Archaea2	School St. Marsh Sediment		12-Jul-07	Antibiotic MPI	Anaerobic					
42	Archaea3	Cedar Swamp deep plant matter		12-Jul-07	Antibiotic MPI	Anaerobic					

	L	M	N	O	P	Q	R	S	T	U
1	R2Result	Restreak3	Restreak3Result	Liquid	LiquidResult	LM image?	cellShape	Thickness	16S sequence	comments2
2	lots	13-Jul-07	radiation from col's	start 7/26	grew quick	image and movie	rods	0.4	Marinobacter, γ	
3						image	rods	0.6		Rods beatout Spiros
4						image	spirilla	0.3		
5						image	cocci, then rods	0.3 cocci, 0.6 rods		formerly cocci, now rods
6		13-Jul-07		start 7/26	grew over 3 days	image	rods, spores	1	B. luciferensis, Firmicutes	SporeFormerOverwhelmed Spirilla
7		26-Jul-07		start 7/26	no growth	yes	spirilla	0.4		
8						image	spirilla	0.4		
9		26-Jul-07				image	rods	0.4	Microbulbifer sp., γ	
10		26-Jul-07				image and movie	mixed	n/a	~ Vibrio diazotrophicus, γ	
11						image and movie	cocci	0.7	~ Vibrio diazotrophicus, γ	
12		26-Jul-07				image	rods, then vibroid	0.7 rods, then 0.6 vibs		
13						image	spirilla	0.4	Sulfurospirillum sp., ε	
14									Sulfurospirillum sp., ε	
15		26-Jul-07				image	spirilla	0.3	~ Arcobacter nitrofigilis, ε	
16		26-Jul-07				movie	spirilla	0.3		
17		26-Jul-07				image and movie	spirilla, rods	~0.45	~ Arcobacter nitrofigilis, ε	has pink bodies at low mag in agar
18									~ Arcobacter nitrofigilis, ε	
19						image	mixed	0.3		
20										
21						image	rods	0.4		
22										
23	some spreaders	25-Jul-07		start 7/26	no growth	image	spirilla	0.5		
24						image and movie	spirilla & rods	0.4		
25						image	spirilla or rods	0.4		
26										
27										
28						image and movie	spirochete	0.25		
29						image	spirochete	0.3		
30										
31						image	spirochete	0.4		
32						iamge and movie	spirilla, rods & spiros			
33	col's	25-Jul-07				image	rods, spores	cells:0.4-0.9; spores: 0.3-0.6	B. firmus, Firmicutes	Also refiltered it over another 0.22 filter over FW LTY on 7/19
34										
35										
36										
37										
38						movie	mixed	~0.7		
39						movie	mixed	0.8 rod, 0.4 spirillum		
40										
41										
42										