Chemotaxis in *Clostridium*

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Abstract

Chemotaxis is a phenomenon whereby motile organisms can sense and respond to compounds found in their immediate environment. *Clostridium* sp. is known to be motile by peritrichous flagella, but it was unknown whether they are chemotactic towards desired substrates. Two *Clostridium* strains isolated from a potato exhibited chemotaxis towards various known growth substrates for saccharolytic Clostridia. A positive chemotactic response was seen in swim plate assays for fructose, xylose, cellobiose, dextrose, and mannitol. Using agar plug assays, a positive chemotactic response was also seen with raffinose, pectin, and starch.

Introduction

Clostridium sp. are found in virtually all environments and are recognized for their ability to ferment several types of organic materials. Many have industrial or medical importance. *C. acetobutylicum* has historically been important in the production of acetone for gunpowder while *C. botulinum*, *C. tetani*, *C. perfringens*, and *C. difficile* have been examined for their toxin production and role in disease (1). Frequently, the saccharolytic Clostridia are involved in the spoilage of meat, dairy products, and the degradation of organics in the environment to acids, alcohols, CO_2, H_2, and some minerals (2-5).

Many motile organisms are chemotactic towards compounds found in their environment at low concentrations. Whether *Clostridium* are chemotactic is an area that has not received much attention. One group has stated that the gut mucus of different animals and humans serves as a chemoattractant for *C. difficile* (6). However, no further work has been done in this field.

With the availability of an increasing number of sequenced genomes, it may be easier to identify genes that are involved in specific cell processes such as chemotaxis. The genome sequences for *C. difficile*, *C. botulinum*, *C. thermocellum*, *C. perfringens*, and *C. acetobutylicum* are currently being completed or have been completed (1,7). Once the annotation procedure is finished for these bacteria, the identification of genes potentially involved in chemotaxis should prove relatively simple. After preliminary examination of the *C. acetobutylicum* genome sequence, several of the genes involved in chemotaxis have been identified.

In this study, we have set out to isolate saccharolytic Clostridia from a potato in order to look for a chemotactic response to several common clostridial growth substrates. We focus on the compounds fructose, xylose, dextrose, cellobiose, lactose, raffinose, mannitol, starch, pectin, and chitin. To look for chemotactic responses, we have employed the use of swim plate assays and agar plug assays.

Materials and Methods

Culture media

A complex medium was used for initial isolation of *Clostridium* strains. This GYT-$CaCO_3$ medium consisted of 1% glucose, 0.5% tryptone, 1% yeast extract, 0.05% sodium thioglycolate and 2% calcium carbonate. When used as a solid medium, 2% agar was added.

A defined medium was also prepared. It consisted of a basal mineral medium (as described in our lab manual) and 0.25% sodium sulfide. To make the liquid anoxic, N_2/CO_2 (90%/10%) gas was bubbled through the liquid for 30 minutes. The solution was then placed in an anaerobic glove box, and 10 ml aliquots were dispensed into Balch tubes. The tubes were sealed with rubber stoppers and aluminum crimps. The tubes were amended prior to use with a 7-vitamin solution and trace elements solution SL10 (as described in our lab manual). Riboflavin was added at a final concentration of 2.5 mg/L. The desired carbon source was added at a final concentration of 5 mM.

Bacterial Strains

To isolate *Clostridium* strains, a potato was rubbed with soil and stabbed with a knife. The potato was placed in a 500 ml beaker and filled with tap water until the level of the water completely covered the potato. The beaker was covered with aluminum foil and placed in a 37° C incubator. When the potato started floating, due to gas production, 2 more days of incubation were allowed before harvesting the potato. A small chunk of liquefied potato was placed in GYT-$CaCO_3$ broth. The tube was overlaid with Vaspar (2:1 parrafin wax to mineral oil) and boiled for 4 minutes. The tube was incubated overnight at 37° C. The liquid was then struck onto GYT-$CaCO_3$ agar plates and incubated at 37° C in anaerobic jars. Isolated colonies were examined under a microscope for the appearance of rod-shaped bacteria containing endospores.

DNA studies

For 16S rDNA sequencing, DNA was extracted from whole colonies using the MoBio Soil DNA Extraction kit. Standard PCR was performed on the DNA samples using the 8F and 1492R universal primers as were used in class procedures. Products were purified using a PCR Purification kit (Qiagen) and sent for sequencing at the University of Connecticut.

Chemotaxis Assays

Chemotaxis was observed using two different techniques (8,9). Swim plates were poured (basal medium, vitamins, trace elements and riboflavin as

previously described) with the addition of 0.5% sodium thioglycolate, 1% MOPS pH 7.2 and a carbon source at 0.5 mM. The final agar concentration was 0.3%. *Clostridium* cultures were grown on the defined medium to early log phase, an OD_{600} of 0.2 to 0.4. The center of the plates was inoculated with 5 μl of the given culture and incubated at 37° C in anaerobic jars. Plates were observed 24-48 hours later and photographed.

Plug assays were also used. Cells were grown to early log phase and harvested. The cells were washed and resuspended in chemotaxis buffer (50 mM potassium phosphate pH 7.0, 20 μM EDTA, 0.05% glycerol) to an OD_{600} of approximately 0.3. Agar plugs contained 2% low melt agar in chemotaxis buffer and a pinch of methylene blue to allow for visualization of the plug. 10 μl drops of the melted agar were spotted on a glass slide. A chamber was made by placing a glass cover slip over top of the plug, supporting it by two plastic strips. The bacterial suspension was then flooded into the chamber to surround the agar plug. Soluble compounds were tested at 5 mM while insoluble compounds were tested at 10% w/v. Control plugs contained no attractant.

Results and Discussion

Identification of two *Clostridium* isolates, named JW1 and Potato 2, used in these experiments was confirmed microscopically by the presence of rod-shaped bacteria containing endospores. The colony morphology seen on GYT-$CaCO_3$ agar plates differed between the two isolates (Fig. 1). The Potato 2 isolate made uniform, flat, off-white colored colonies on the plates and did not produce much acid (clearing around the colony). The JW1 strain, on the other hand, produced an extremely mucoid, sticky, round colony with a pinkish hue. These colonies also did not produce much acid. An initial attempt to characterize the pinkish color of the JW1 strain proved unsuccessful. Both cultures produced rod-shaped bacteria with endospores (Fig. 1).

The 16S rDNA analysis was inconsistent. Although bands were obtained for both JW1 and Potato 2 in the PCR amplification of the 16S gene, sequence was only obtained for the JW1 strain. A BLAST comparison of the sequence returned two hits of exactly the same similarity to *Clostridium acetobutylicum* and *Clostridium beijerinckii*. Given a more complete sequence from the PCR, it may have been easier to identify the isolate. Both *C. acetobutylicum* and *C. beijerinckii* are known as saccharolytic Clostridia. Despite the inability to definitively identify the isolate, this result supports the goal of examining chemotaxis in the saccharolytic Clostridia.

A chemotactic response was observed in the swim plates by the formation of a ring of bacteria on the plate. The *Clostridium* cells created their own concentration gradient of the given substrate. The cells grew in the center of the plate, consuming all of the carbon source found there. In search of a higher concentration of the substrate, the cells swam out from the center of the plate in the formation of a ring. The swim plates worked well for the majority of the

soluble substrates including dextrose, fructose, mannitol, xylose, and cellobiose (Fig. 2). Rings were typically evident after 16-24 hours. An incubation period of 48 hours was necessary for the plates containing mannitol. Both strains showed the same result for these 5 substrates tested except there was an inconclusive result for the Potato 2 strain growing on xylose.

Growth curves of the two strains were completed for dextrose, fructose, cellobiose, and xylose (Table 1). The cultures were grown on the defined medium plus vitamins and metals with the addition of 5 mM carbon source. On average, both strains had a doubling time of 1-2 hours for the given saccharide substrates.

Table 1. Doubling time of the two isolates reported in hours.

	Cellobiose	Dextrose	Fructose	Xylose
JW1	1.9	1.44	1.55	ND*
Potato 2	1.1	0.97	1.89	2.02

*Not Determined

The *Clostridium* isolates also showed a chemotactic response in the agar plug assays. A positive response was observed as the formation of a ring of bacteria around and touching the agar plug containing attractant compared to an agar plug containing no attractant. Diffusion of the substrate from the plug into the surrounding environment creates a gradient of the substrate to which the cells are attracted. The plug assays were used to test the soluble compounds lactose, pectin, and raffinose in addition to the insoluble substrates starch and chitin.

A positive response was seen with the JW1 and Potato 2 strains for both starch and pectin. The Potato 2 strain also gave a positive response for raffinose whereas an inconclusive result was seen for the JW1 isolate. After several attempts to see a result for lactose and chitin, none was seen for either isolate (Fig. 3).

A composite list of substrates tested and the chemotactic response is shown in Table 2. These results seem consistent with what one might expect for saccharolytic Clostridia that were isolated from a potato. Few *Clostridium* species are able to utilize chitin, the main constituent found in insect exoskeletons. *C. sporogenes* is one of a few Clostridia that can use chitin (10). Based on the BLAST of the 16S rDNA sequence for these isolates, *C. sporogenes* did not have a significant match with the isolated strain. No response was seen with lactose in either the swim plate assays or the agar plug assays. The plates were incubated for 48 hours and still no response was evident. Perhaps a longer

incubation period or a change in the substrate concentration is needed to verify whether or not an actual response is possible with this substrate.

Although the agar plug assays worked well for many substrates, one drawback to these assays is the presence of oxygen in the system. The *Clostridium* are strict anaerobes. Once the cells are harvested for use in the plug assays, there is oxygen present in the system. Typically, the assays were only conducted for about 10-15 minutes before a picture was taken to document whether or not a positive response was evident. This amount of time was chosen in order to limit exposure to oxygen but still see a chemotactic response. A more clear cut accumulation of cells may have been possible if the assays were conducted for longer periods of time. In addition, the *Clostridium* cells are only motile during early logarithmic phase. Perhaps cells should have been concentrated to an OD_{600} of approximately 0.6 in order to see a greater response.

There are several species of *Clostridium* that are sequenced and/or currently being sequenced. One species that has already been completed is *C. acetobutylicum* (1). A quick examination of the annotated genes from this species reveals the presence of a *cheY* homolog, *cheA*, *cheW*, *cheR* and an MCP (11). Based on the presence of these genes, one might assume the bacterium is chemotactic. The results of these tests support this hypothesis. The swim plates and agar plug assays both definitively show that chemotaxis is present in the JW1 and Potato 2 strains isolated. A next step might be to locate these genes and attempt to mutagenize those involved in chemotaxis. A failure to respond to a concentration gradient in a mutant would lead to a further understanding of the chemotaxis machinery in *Clostridium*. These data serve as a promising starting point for the continued characterization of chemotaxis in *Clostridium*.

References:

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Figure 1. Dissecting scope images and wet mounts of JW1 and Potato 2 isolates.

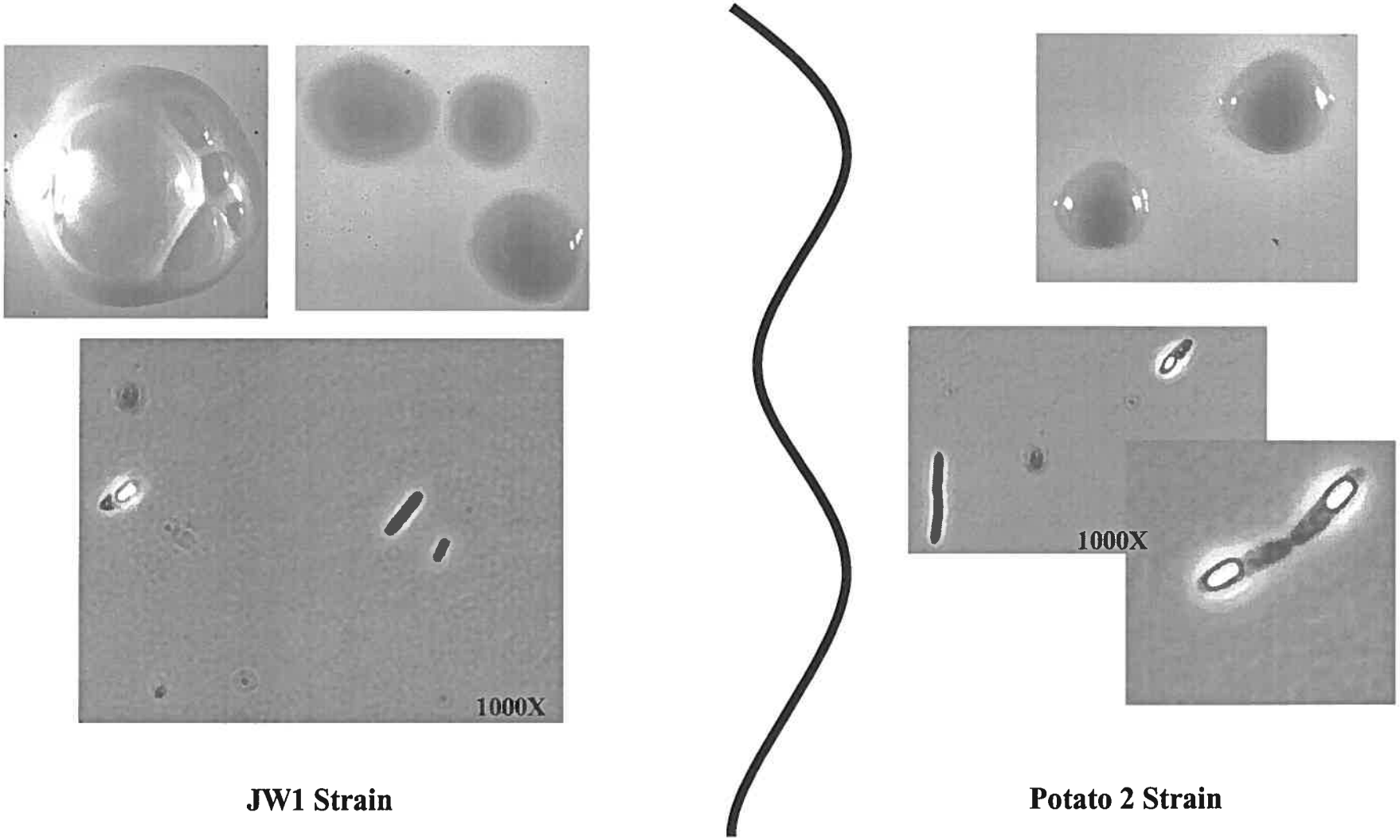
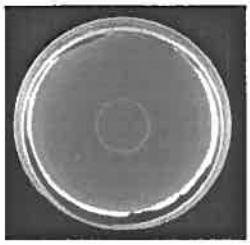
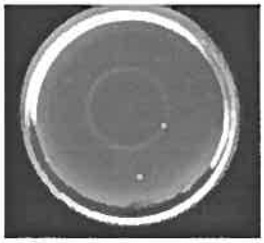


Figure 2. Swim plate assay results. All carbon sources used at 0.5 mM. Plates were incubated anaerobically at 37° C for 16-48 hours.

Fructose

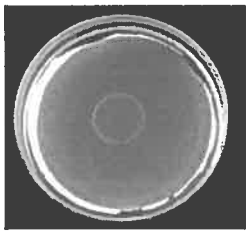


JW1

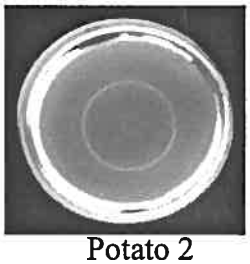


Potato 2

Dextrose

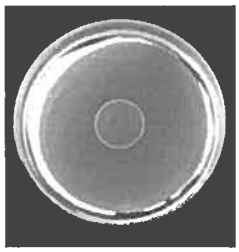


JW1

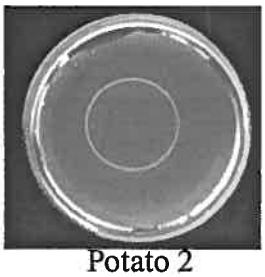


Potato 2

Cellobiose

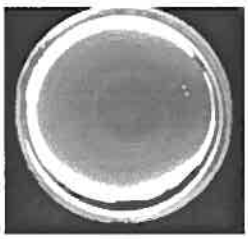


JW1



Potato 2

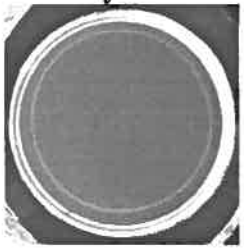
Mannitol



JW1

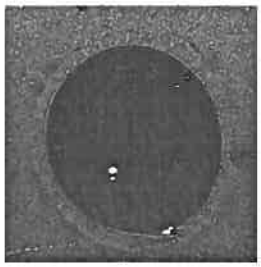
Potato 2

Xylose

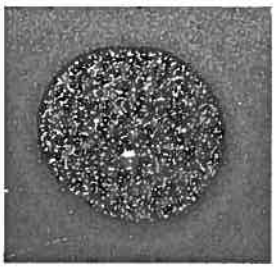


JW1

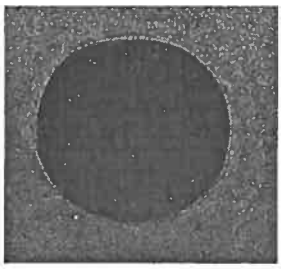
Figure 3. Agar plug assays with the Potato 2 strain. Agar plugs are 2% low melt agar with methylene blue. Those plugs containing attractant are 5 mM for soluble substrates and 10% (w/v) for insoluble substrates. Washed cells were resuspended in chemotaxis buffer and flooded into the plug chamber. Pictures were visualized after 10 minutes.



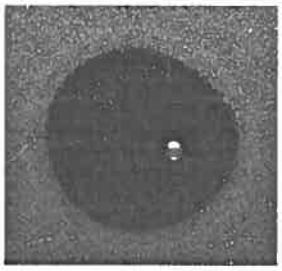
No attractant



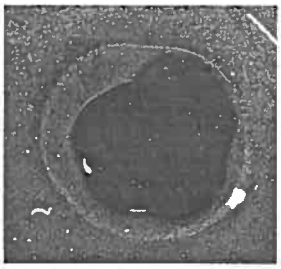
10% starch



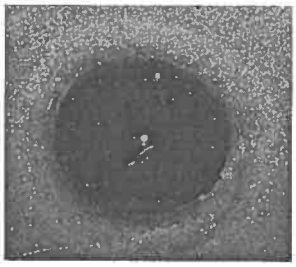
No attractant



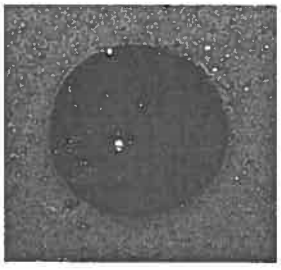
5 mM Raffinose



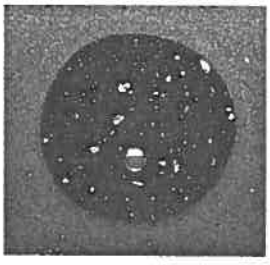
No attractant



10% pectin



No attractant



10% chitin

Table 2. Comprehensive results from chemotaxis assays. A + denotes a positive response and chemotaxis. A - denotes no noticeable chemotactic response. ND is not determined. Swim plate data are designated by "swim" and agar plug data by "plug".

	JW1	**Potato 2**
Cellobiose	+ swim	+ swim
Chitin	-	-
Dextrose	+ swim	+ swim
Fructose	+ swim	+ swim
Lactose	-	-
Mannitol	+ swim	+ swim
Pectin	+ plug	+ plug
Raffinose	+ plug	+ plug
Starch	+ plug	+ plug
Xylose	+ swim	ND