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The use of genetic probes to detect microorganisms in biomining operations

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SUMMARY

Microorganisms are currently used for the recovery of copper from mining dumps of low-grade ore. One of the most important microorganisms involved in copper-solubilization is *Thiobacillus ferrooxidans*, although many other microbial genera are also thought to be implicated. A mining dump poses some special problems for the industrial microbiologist because it represents a non-sterile and heterogeneous substrate. Consequently, to enhance our knowledge of the role of microorganisms in metal recovery we must identify the indigenous microorganisms and understand their respective contributions to the process. In addition, when a superior strain of microorganism is developed in the laboratory, by genetic engineering or by other means, we must have a method to evaluate the maintenance of such a strain in the mining dump. In this paper, we describe DNA homology studies, using dot blot and Southern blot analysis of hybridizations of both whole genomic DNA and cloned DNA sequences, to identify and enumerate several bioleaching microorganisms. We demonstrate that it is possible to identify different species of microorganisms and, in one case, to discriminate between different strains of a single species. It is also possible to identify and quantitate certain species in a mixed culture. DNA hybridization analysis has several advantages over the more conventional bacteriological methods of identification, especially in a complex bioleaching situation.

INTRODUCTION

About 10% of the copper in the U.S.A. is obtained from dumps of low-grade ore by a process termed bioleaching in which microorganisms play an active part in the metal-recovery process. An example of a bioleaching dump is shown in Fig. 1. Such a dump contains rocks with too little copper to warrant economic recovery by conventional techniques such as smelting. Instead, most mining companies sprinkle dilute sulfuric acid on top of these dumps to encourage the growth of indigenous microorganisms. These microorganisms promote the solubilization of metals in the rocks by two methods: directly by oxidizing them and indirectly by producing ferric ions via the oxidation of ferrous iron. Ferric ion is a strong chemical oxidant of certain metals. Many of the microorganisms are also

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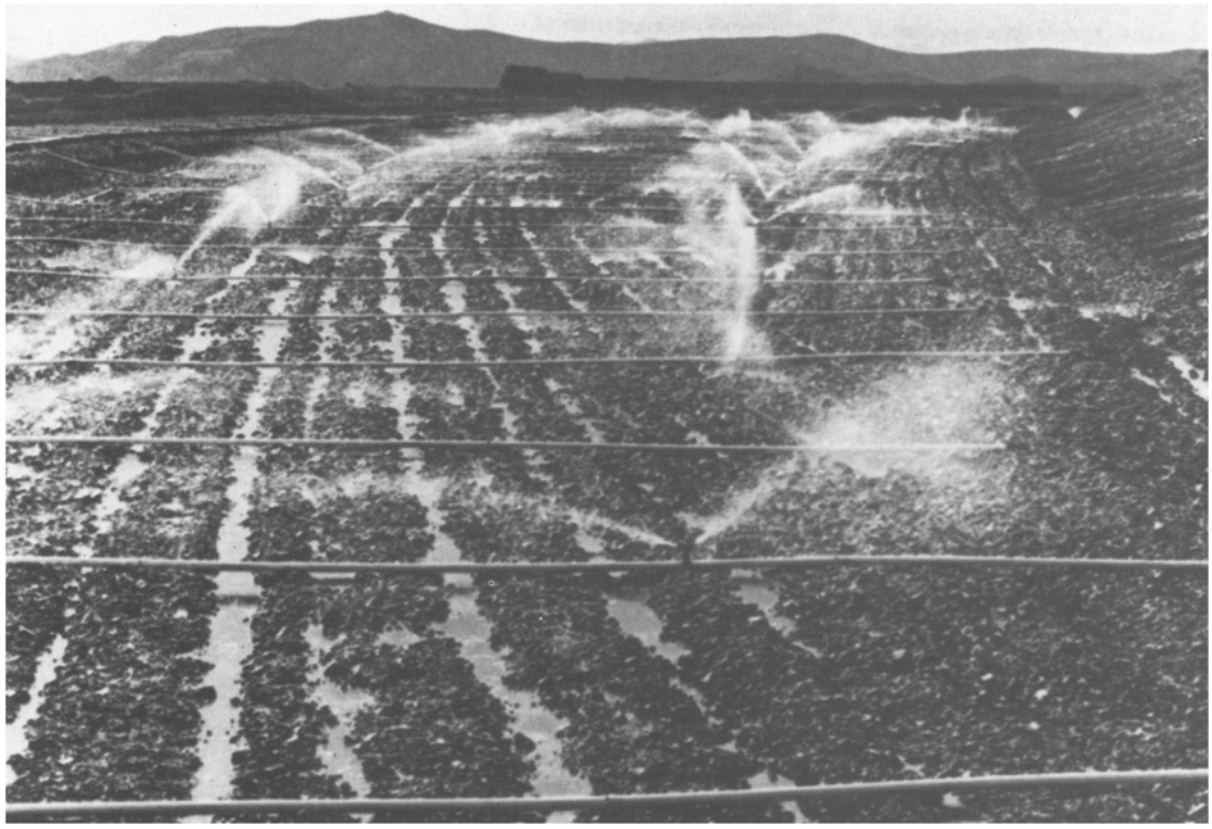

Fig. 1. A dump-leaching operation for the recovery of copper from waste material. Dilute acid is being sprinkled to promote the growth of acidophilic microorganisms such as *Thiobacillus ferrooxidans* and to enhance the solubilization of the oxidized copper.

capable of oxidizing reduced sulfur compounds such as pyrite. One of the by-products of sulfur metabolism, sulfuric acid, promotes the solubilization of the oxidized metals. *Thiobacillus ferrooxidans* is one of the best characterized of these bioleaching microorganisms. However, there are many others that may be very important in the bioleaching process such as *Thiobacillus thiooxidans, Leptospirillum, Gallionella,* thermophilic *'Thiobacillus*-like' organisms, and various heterotrophic bacteria. Although the relative importance of these microorganisms for metal solubilization is not completely understood, it is clear that the process is a complex one.

There are several interesting problems that are being addressed by the industrial microbiologist in order to understand more fully the bioleaching process and, in the long term, to improve its efficiency. A bioleaching operation, unlike a fermentation tank, cannot be sterilized and subsequently inoculated with a pure culture. As a consequence, there is an absolute requirement to identify and enumerate the naturally occurring microorganisms and to evaluate their respective roles in metal solubilization. This is not a simple task, given the variety of such organisms and the difficulties in identifying them.

The problem is compounded by several additional attributes of a bioleaching operation. The species of microorganisms involved in metal solubilization may differ extensively throughout a leach dump and between dumps due to variability in the substrate, pH, temperature, O_2 and CO_2 concentrations, etc. Also, changes may occur over time within a dump because of the ecological succession of microorganisms that takes place as the dump

matures. This means that many samples must be taken from different parts of the dump and over a period of time. Also, information on the identification and enumeration of microorganisms present in one type of dump may not be valid for another dump, so the process must be repeated.

Another problem that the industrial microbiologists will have to address is how well a superior laboratory strain is maintained when it is added to a dump. A similar problem will be encountered in other situations where laboratory strains are used in environmental settings such as the use of microorganisms to remove polychlorinated biphenyls or heavy metals from industrial wastes.

In this paper we describe a method that relies on DNA hybridization of cloned DNA sequences as a means of identifying and enumerating several species of bioleaching microorganisms. In addition, we show that cloned DNA sequences can be used to distinguish between strains of the single species *T. ferrooxidans*.

MATERIALS AND METHODS

Cells and culture conditions

Acidophilic autotrophs.
Four strains of *Thiobacillus ferrooxidans* were maintained in 9K minimal salts medium plus $FeSO_4$ [14]. Two of these strains were obtained from the American Type Culture Collection (ATCC 19859 and ATCC 33020) and the other two strains (ATCC 13361 and Torma) were gifts from H. Ehrlich and A. Torma, respectively.

Neutrophilic autotroph. Thiobacillus thioparus (ATCC 8158) was maintained in S-6 medium + 1% $Na_2S_2O_3$ [1].

Acidophilic heterotrophs. Two strains of *Thiobacillus acidophilus* (ATCC 27807 and DSM 700, from the Deutsche Sammlung von Mikroorganismen) and *Acidiphilium organovorum* (ATCC 43141) (isolated in our laboratory) were maintained in 9K minimal salts + 1% glucose. *Acidiphilum cryptum* (a gift from A. Harrison) was maintained in 9K minimal salts + 0.1% glucose [7].

Neutrophilic heterotroph. Thiobacillus novellus (ATCC 8093) was maintained in Nutrient broth (BBL).

DNA preparation

Total DNA was prepared by a modification of the method of Blin and Stafford [3]. Cells were collected by centrifugation and resuspended in 2 ml of 0.4 M EDTA + 0.2 M Tris (pH 9.2), and added dropwise to 20 ml of 0.4 M EDTA + 0.2 M Tris (pH 9.2) pre-heated to 65°C. Cells were lysed by the drop-wise addition of 20% SDS to a final concentration of 1% SDS and maintained at 65°C for 15 min. The lysed cells were cooled to 50°C and Proteinase K (Beckman) was added to a final concentration of 500 μg/ml. Incubation was continued at 50°C for approx. 18 h (during which time another similar aliquot of Proteinase K was added). Cell lysates were extracted with phenol/chloroform (1:1) five or six times and total DNA was purified by one round of CsCl buoyant density centrifugation.

Generation of recombinant plasmids

Total DNA from *T. ferrooxidans* (ATCC 19859) was subjected to partial digestion with *Mbo*I and fragments corresponding to 6–10 kb were isolated by sucrose gradient centrifugation. These fragments were inserted into the *Bam*HI site of pBR322 and transformed into *Escherichia coli* C600. Recombinant plasmids were screened by the rapid heating method [9] and purified by the rapid boiling procedure [5].

Nick translations

All nick translations were performed according to the manufacturer's instructions using a nick translation reagent kit (Bethesda Research Labs, Gaithersburg, MD) and α-^{32}P-labeled dCTP (800 Ci/mmol) (Amersham, Inc., Arlington Heights, IL). DNA was routinely labeled to 10^8 cpm per microgram.

Southern blots and dot blots

Southern blots [13] and dot blots were carried out using GeneScreen™ Hybridization Transfer Membrane (New England Nuclear, Inc., Boston,

MA) essentially according to the manufacturer's recommendations. Hybridization was done at Tm-32°C and the most stringent wash was done at Tm-37°C. Autoradiography was for different lengths of time on Kodak X-Omat X-ray film.

RESULTS AND DISCUSSION

DNA hybridization analysis has been successfully employed for the identification of a number of species and strains of microorganisms, such as *Salmonella* spp. in foods [6], members of the Enterobacteriaceae [4], *Bacteriodes thetaiotaomicron* strains [12] and *Acholeplasma* spp. [2]. Solution hybridization has been used to determine the extent of genetic diversity between *T. ferrooxidans* and *T. thiooxidans* and between strains of *T. ferrooxidans* [8]. In the present paper we have extended these studies to include dot blot and Southern blot hybridization analyses both between *T. ferrooxidans* and several other species, and within *T. ferrooxidans* strains.

Fig. 2 shows a dot blot where total DNA from several different microorganisms has been applied to a membrane and then probed with ^{32}P-labeled DNA prepared from *T. ferrooxidans* ATCC 19859. Although *T. ferrooxidans* ATCC 19859 contains a large (> 30 kb) plasmid, all the DNA probes derived from this strain in this study were of genomic origin (unpublished results). The probe DNA hybridized efficiently with all four strains of *T. ferrooxidans* (Fig. 2, lanes A–D). However, there was very little hybridization to two species of *T. acidophilus* (Fig. 2, lanes E, F), *T. novellus* (Fig. 2, lane H), two species of acidophilic heterotrophs (Fig. 2, lanes I, J), and *E. coli* (Fig. 2, lane K). *T. thioparus* (Fig. 2, lane G) showed a small degree of homology.

The most striking observations were the lack of hybridization between *T. ferrooxidans* ATCC 19859 and *T. novellus* and the relatively small degree of homology with *T. thioparus*. This indicates considerable sequence divergence and strongly supports the idea that neither *T. novellus* nor *T. thioparus* belong in the same genus as *T. ferrooxidans* [8,10,15].

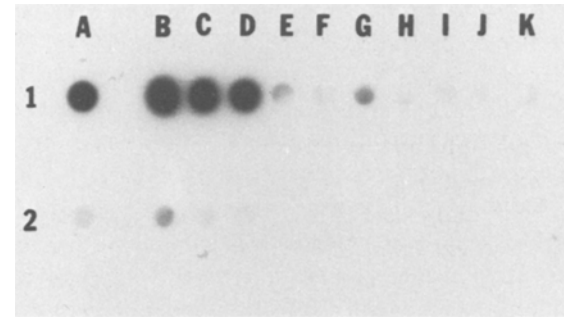

Fig. 2. Dot blot of genomic DNAs. Two concentrations (100 ng and 1 ng) of genomic DNA from each organism were applied to the membrane and probed with $\approx 2 \cdot 10^7$ cpm of ^{32}P-labeled genomic DNA from *T. ferrooxidans* ATCC 19859. Lane A, *T. ferrooxidans* ATCC 19859; lane B, *T. ferrooxidans* strain Torma; lane C, *T. ferrooxidans* ATCC 33020; lane D, *T. ferrooxidans* ATCC 13661; lane E, *T. acidophilus* ATCC 27807; lane F, *T. acidophilus* (DSM 700); lane G, *T. thioparus*; lane H, *T. novellus*; lane I, *A. cryptum*; lane J, *A. organovorum* ATCC 43141; lane K, *E. coli* C600.

Fig. 3 shows a dot blot where DNAs from several different microorganisms have been applied to a membrane filter and then probed with ^{32}P-labeled DNA prepared from an in-house strain of acidophilic heterotroph *A. organovorum* ATCC 43141. Acidophilic heterotrophs are common in bioleaching operations and may be important in the process of metal solubilization due to an apparent symbiosis with *T. ferrooxidans*. Strain TFC shows little homology to *T. ferrooxidans* (Fig. 3, lanes 6, 7), but

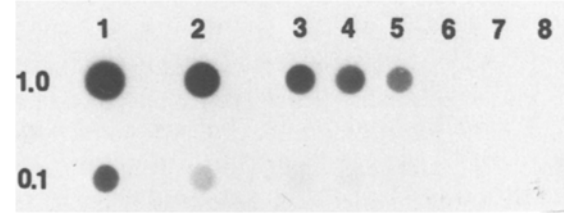

Fig. 3. Dot blot of DNA:DNA hybridization. The DNA from eight different bacteria (in the amounts indicated on the left) were applied to the membrane and hybridized with 1 μg of ^{32}P-labeled DNA from strain TFC. The numbers at the top of each column correspond to DNA from the following bacteria: 1, *A. organovorum* ATCC 43141; 2, *A. cryptum*; 3, *T. acidophilus* ATCC 27807; 4, *T. acidophilus* DSM 700; 5, *T. novellus*; 6, *T. ferrooxidans* ATCC 13661; 7, *T. ferrooxidans* ATCC 33020; 8, *E. coli* strain B.

does exhibit extensive homology to two other acidophilic heterotrophs, *Thiobacillus acidophilus* (Fig. 3, lanes 3, 4) and *Acidiphilium cryptum* (Fig. 3, lane 2).

The results demonstrate that genomic DNA hybridization can be used to distinguish between certain bacterial species active in the bioleaching process. For example, it can unambiguously distinguish between any test strain of *T. ferrooxidans* and the acidophilic heterotrophs. But, what about a case, for example, where one needs to distinguish between the different strains of *T. ferrooxidans* in a solution derived from a bioleaching operation? The extensive homology between these strains precludes the use of genomic DNA homology testing.

In order to discriminate between microorganisms at the subspecies level, cloned DNA sequences can be used as hybridization probes. For example, Fig. 4 shows a Southern blot using a cloned sequence derived from *T. ferrooxidans* ATCC 19859 as a probe. The sequence has excellent homology to all four strains of *T. ferrooxidans* examined (Fig. 4, lanes 3–6), but exhibits no homology to any of the other DNAs. This contrasts with the data shown in Fig. 2 where total genomic DNA hybridization was unable to discriminate unambiguously between *T. ferrooxidans* and *T. thioparus*. In addition, the probe can clearly distinguish between three of the four strains of *T. ferrooxidans* by virtue of the different restriction fragments that are detected. This is termed restriction fragment length polymorphism (RFLP). For example, although *T. ferrooxidans* 19859 (Fig. 3, lane 3) has the same restriction fragment pattern as *T. ferrooxidans* strain Torma (Fig. 3, lane 4) both can be differentiated from *T. ferrooxidans* ATCC 33020 and ATCC 13661 (Fig. 4, lanes 5 and 6, respectively).

The Southern blot technique is well suited for this type of analysis because it permits the detection of restriction fragment length polymorphisms in addition to differences in the extent of hybridization. However, it is more difficult to execute than the dot blot. Therefore, we addressed the question of whether a cloned sequence could be used in a dot blot assay to distinguish between species and between strains of a single species. A number of

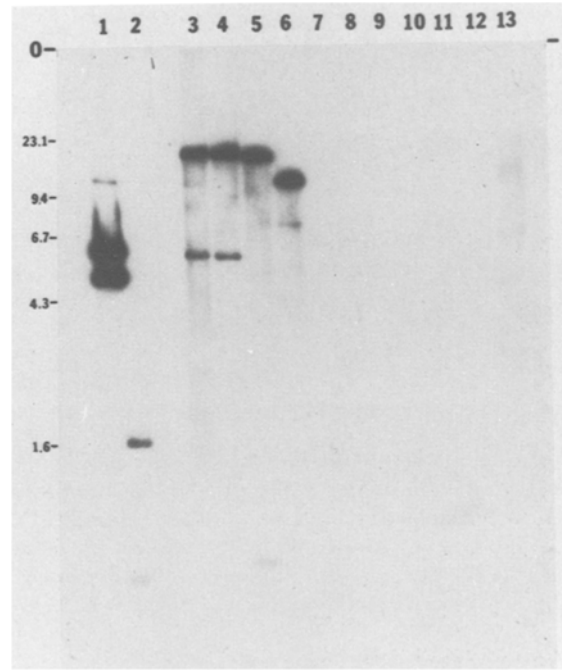

Fig. 4. Southern blot of *Eco*RI-digested genomic DNAs hybridized with a cloned sequence (termed pTf 10) derived from *T. ferrooxidans* ATCC 19859. Approximately 1.5 μg of *Eco*RI-digested genomic DNA from each organism was electrophoresed in a 0.8% agarose gel and transferred to a nylon membrane. Lane 1, pTf 10; lane 2, molecular weight size marker; (1 kilobase ladder (BRL, Inc.)); lane 3, *T. ferrooxidans* ATCC 19859; lane 4, *T. ferrooxidans* strain Torma; lane 5, *T. ferrooxidans* ATCC 33020; lane 6, *T. ferrooxidans* ATCC 13661; lane 7, *T. acidophilus* ATCC 27807; lane 8, *T. acidophilus* DSM 700; lane 9, *T. thioparus*; lane 10, *T. novellus*; lane 11, *A. cryptum*; lane 12, *A. organovorum* ATCC 43141; lane 13, *E. coli* C600.

cloned DNA sequences were hybridized individually to DNA from several *Thiobacillus* species and acidophilic heterotrophs. A probe (termed pTf 1000) was selected that hybridized to *T. ferrooxidans* but not to the other species of *Thiobacillus* or the acidophilic heterotrophs (data not shown). pTf 1000 was then used as a hybridization probe in the dot blot (Fig. 5). The probe can readily detect 10^{-4} μg DNA from *T. ferrooxidans* ATCC 19859 (Fig. 5, row A) and strain Torma (Fig. 5, row B). It can detect 10^{-5} μg DNA in a longer exposure of the autoradiogram. If one makes the assumption that the genome size of *T. ferrooxidans* is the same as

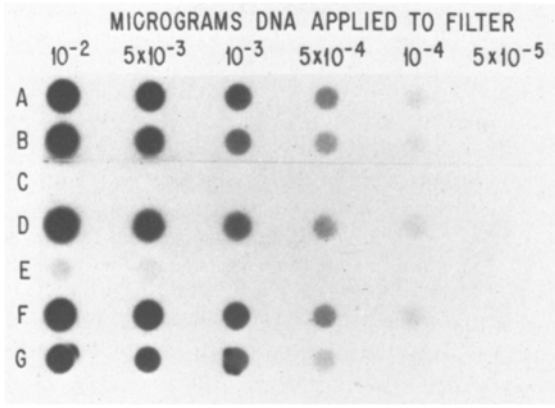

Fig. 5. Dot blot of genomic DNAs hybridized with a cloned sequence (termed pTf 1000) derived from *T. ferrooxidans* ATCC 19859. Rows contain different genomic DNAs. Columns contain different concentrations of DNA. Row A, *T. ferrooxidans* ATCC 19859; row B, *T. ferrooxidans* strain Torma; row C, *T. acidophilus* ATCC 27807; row D, equal weights of *T. ferrooxidans* ATCC 19859 and *T. acidophilus* ATCC 27807; row E, *T. ferrooxidans* ATCC 13661; row F, equal weights of *T. ferrooxidans* ATCC 19859 and lambda phage; row G, pTf 1000.

that of *E. coli* then we could detect the presence of approximately 10^5 cells. A second observation is the lack of detectable hybridization to *T. acidophilus* (Fig. 5, row C), which agrees with the Southern blot data. Also, the extent of hybridization of probe pTf 1000 to *T. ferrooxidans* ATCC 13361 is equivalent in intensity to only 1% of the hybridization to the parental DNA of *T. ferrooxidans* ATCC 19859. Therefore, probe pTf 1000 could be used to discriminate effectively between these two strains by dot blot analysis. Moreover, the addition of non-homologous *T. acidophilus* DNA to the DNA of *T. ferrooxidans* ATCC 19859 does not apparently interfere with hybridization (Fig. 5, row D). Therefore, it should be possible to detect specific strains present in mixed cultures without interference by non-homologous DNA.

We have extended our survey using additional probes (data not shown) to increase the versatility of the technique. What one would like to have is a series of probes that can identify any relevant biomining microorganisms. In addition, one would also like to have a set of probes that could distinguish between the various *T. ferrooxidans* strains. This would be especially useful if specific strains carrying desirable traits, such as enhanced iron oxidation or metal resistance, could be identified by their respective restriction fragment length polymorphisms.

If a laboratory strain containing a unique sequence is placed in a bioleaching operation, its survival relative to the natural population of microorganisms could be monitored by dot blot analysis using the unique sequence as a probe. If there is no such sequence, then a unique marker DNA sequence could be introduced into the genome by genetic engineering. But such a sequence must not interfere with the growth and metabolism of the cell and should be stably maintained. Such an exercise would be a very useful application of genetic engineering.

The dot blot procedure, utilizing cloned DNA sequences as probes, has the necessary discrimination and sensitivity to answer the questions posed earlier. Namely, how can one identify and enumerate the microorganisms in a bioleaching operation and how could one monitor the fate of a laboratory strain intoduced to a bioleaching operation? At the very least, the technique, in conjunction with the Southern blot analysis, will provide a very powerful tool for establishing a classification system for bioleaching microorganisms based on genetic relationships. Such a classification needs to be developed because of the apparent genetic diversity that exists between species in the genus *Thiobacillus* [10].

But will the technique ever become more than an analytical procedure used in the laboratories of microbiologists and molecular biologists? Obviously, additional probes specific for other biomining organisms need to be generated. For example, probes for species and strains from the genera *Sulfolobus* and *Leptospirillum* genera and so on. Also, it is desirable that analytical probes should become generally available to permit uniform standardization. In addition, a considerable amount of work needs to be done to simplify the protocols and to determine their general applicability in a number of experimental situations. For example, it would be advantageous if the dot blot procedure could be done on lysed bacterial cells without requiring the steps involved in the purification of the bacterial

DNA. An alternative to the use of ^{32}P would be desirable where experience in handling radioactive material is limited. Such an alternative DNA-labeling procedure, which utilizes biotinylated probes, is available [11]. An additional advantage of using biotinylated probes is that they are stable for many months, whereas ^{32}P-labeled probes decay with a half life of 14 days.

Finally, at the present time, it requires th expertise and support equipment of a molecular biologist to construct the experiments and interpret the results of DNA homology studies. The technology would become more attractive to mining companies if automated equipment were available for carrying out the hybridizations and the quantitations of the results, using protocols that could be carried out easily and routinely by a technician. Such equipment and protocols are being developed by companies interested in DNA hybridization as a tool to evaluate genetic disorders in human. This type of equipment should become available for the biomining community.

ACKNOWLEDGEMENTS

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