APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1982, p. 135–140
0099-2240/82/070135-06$02.00/0

Vol. 44, No. 1

Accumulation of Amino Acids in *Rhizobium* sp. Strain WR1001 in Response to Sodium Chloride Salinity

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Received 19 October 1981/Accepted 5 March 1982

Rhizobium sp. strain WR1001, isolated from the Sonoran Desert by Eskew and Ting, was found to be able to grow in defined medium containing NaCl up to 500 mM, a concentration approaching that of sea water. Therefore, it is a valuable strain for studying the biochemical basis of salt tolerance. Intracellular free glutamate was found to increase rapidly in response to osmotic stress by NaCl. It accounted for 88% of the amino acid pool when the bacterium was grown in 500 mM NaCl. The role of glutamate dehydrogenase in glutamate biosynthesis was examined in several *Rhizobium* strains. Both NADH- and NADPH-dependent glutamate dehydrogenase activities in various *Rhizobium* strains were observed. The range of activity differed considerably depending on the particular strain. KCl (500 mM) did not stimulate glutamate dehydrogenase activity, as reported in a number of bacterial strains by Measures. The low activity of glutamate dehydrogenase in *Rhizobium* sp. strain WR1001 apparently cannot fulfill a biosynthetic function of glutamate formation in response to medium NaCl concentrations.

Salinity has become an ever-increasing problem in irrigated agriculture (10, 21). Most *Rhizobium* strains which nodulate important crops such as soybean, pea, and clover are very sensitive to salt (14, 24, 25, 27, 30). Several reports have established that progressive increases in salt concentration adversely affect inoculum viability and the symbiotic nitrogen fixation rate (1, 29). Graham and Parker (14) recorded that some strains of *R. meliloti* were tolerant of 2% NaCl but none of the 19 strains belonging to other *Rhizobium* groups could grow at this concentration. They concluded that 3% NaCl was critical for *Rhizobium* spp.

Rhizobium sp. strain WR1001, which nodulates the legume plant, *Prosopis* spp. (mesquite), was isolated from the Sonoran Desert (12). This bacterial strain was found to be capable of growth in defined medium containing 3% NaCl (~500 mM), a level approaching that of sea water (S.-S. Hua, P. E. Felker, G. Fuller, A. Stafford, and H. G. Bayne, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, K176, p. 156). Therefore, it is a useful strain for studying the biochemical basis of salt tolerance in *Rhizobium* sp.

The metabolic activities of microorganisms vary considerably with changes in their growth environments. Environmental changes are most likely to affect the intracellular concentrations of the low-molecular-weight pool constituents. One of the parameters affecting microbial physiology is the medium osmolarity. It has been well established that glutamate is predominant in the intracellular amino acid pool in gram-negative bacteria and that it is highly sensitive to changes in growth conditions. For example, NaCl concentration in the medium has a gross effect on the intracellular levels of glutamate in many bacterial species (3, 4, 17, 26). Free glutamate increases in most gram-negative bacteria in media containing high NaCl concentrations, whereas gram-positive bacteria accumulate proline. Proline and γ-aminobutyrate have also been found to increase in some gram-negative bacteria (17, 26). It seems then that under osmotic stress, the fundamental response of gram-negative bacteria is an increase in the amino acid pool, particularly glutamate.

The enzyme glutamate dehydrogenase (GDH; EC 1.4.1.4) is an important link between the tricarboxylic acid cycle (Krebs cycle) and the metabolism of amino acids. Measures (17) has demonstrated that GDH in cell-free extracts of several bacteria is activated by 500 mM K^+ up to 10-fold more than that without K^+ in the direction of glutamate formation, but not in the reverse reaction. Intracellular K^+ concentration was found to increase as a result of the addition of NaCl to the medium. Therefore he hypothesized that rapid changes in the intracellular free glutamate content of bacteria may be mediated by changes in the cellular activity of this enzyme stimulated by higher concentrations of K^+. Epstein and Laimins (11) have provided experimental evidence that K^+ transport is regulated by osmotic stress.

The success of symbiotic biological nitrogen fixation in saline soil depends on the survival

and growth of the rhizobia introduced (6, 28). Therefore, salt tolerance in *Rhizobium* spp. is a desirable agronomic trait. Some natural isolates of rhizobia are reported to be salt tolerant (2, 14, 30, 31). However, the biochemical and physiological bases of salt tolerence in *Rhizobium* spp. have not been investigated. We used the salt-tolerant *Rhizobium* sp. strain WR1001 as the experimental subject. The principal aim of the study was to examine the amino acid pool of *Rhizobium* sp. strain WR1001 grown in media containing different NaCl concentrations. The role of GDH in glutamate biosynthesis was assessed. Glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (GOGAT; EC 2.6.1.53) activities of several *Rhizobium* strains were also assayed.

MATERIALS AND METHODS

Bacterial strain. Mesquite *Rhizobium* was kindly provided by P. Felker, Texas A & I University, Kingsville. It was designated *Rhizobium* sp. strain WR1001, and we use that designation below. *R. japonicum* 110 was kindly provided by D. E. Weber, U.S. Department of Agriculture, Beltsville, Md. *R. meliloti* 2011 was kindly provided by J. Denárie, Centre National de la Recherche Agronomique, Versailles, France. *R. meliloti* 41 (AK631) was kindly provided by A. Kondorosi, Hungarian Academy of Sciences, Szeged, Hungary. *Rhizobium* sp. strain CB756 was kindly provided by Z. Shamsuddin, Murdoch University, Murdoch, Western Australia.

Growth conditions. The bacterium was grown in minimal nitrate-mannitol medium containing (grams per liter): Na_2HPO_4, 0.26; Na_2SO_4, 0.03; KNO_3, 0.6; $FeCl_3 \cdot 6H_2O$, 0.01; $MgCl_2 \cdot 6H_2O$, 0.1; $CaCl_2 \cdot 6H_2O$, 0.1; D-mannitol, 2. Thiamine and biotin were added at a concentration of 1 mg/liter, and the pH was adjusted to 7.0. In glutamate-manitol medium, 0.09% (wt/vol) glutamate was used to replace nitrate. For growth in different NaCl concentrations, sodium chloride was added to the minimal medium to the desired amount. An early stationary-phase culture was used to inoculate the fresh medium. Growth was monitored by turbidity measurements with a Klett-Summerson colorimeter at 540 nm.

Analytical procedures. The intracellular amino acid pools of *Rhizobium* sp. strain WR1001 grown in various salt concentrations were determined by the following procedure. The bacterium was grown in minimal nitrate-mannitol medium containing 0 to 500 mM NaCl until late-logarithmic phase. For each culture, 100 ml of cells was centrifuged at 17,000 × *g* for 15 min. The pellets were washed and suspended in isotonic salt solutions and then recentrifuged. The amino acids from the resulting pellets were extracted by suspending in 1 ml of 5% trichloroacetic acid (TCA) at 0°C for 2 h or overnight with occasional blending in a Vortex mixer. The suspensions were then centrifuged at 27,000 × *g* for 30 min. The supernatants obtained were used for amino acid analysis with a Durrum amino acid analyzer, model D-500, with a single column (1.75 mm by 48 cm) packed with Durrum DC-4A resin. Citrate buffers of pH 3.25, 4.25, and 7.90 were used. Amino acid concentrations were determined by reaction with ninhydrin reagent and photometric measurement at 440 and 590 nm. Analysis time was 90 min.

Total amino acid concentrations in the 5% TCA extracts were determined by the method of Rosen (22) before amino acid analysis. Proline concentrations were estimated by the acid ninhydrin procedure (15).

Preparation of crude cell extracts. Late-log-phase cells (150 to 200 Klett units) were pelleted and washed once in 50 mM imidazole buffer (pH 7.0). For GDH and GOGAT assays, the resulting pellets were suspended in 50 mM imidazole buffer (pH 7.5) containing 1 mM mercapto-ethanol to 1/20 or 1/40 of the original cell culture volume. For GS assays, the pellets were suspended in 10 mM imidazole buffer (pH 7.0) containing 1 mM $MnCl_2$ to 1/20 of the original culture volume. Extracts were prepared by ultrasonic disruption for a total of 3 min in 15-s pulses at 0°C. Extracts were centrifuged at 27,000 × *g* for 20 min at 4°C, and the supernatant was used for enzyme assays.

Enzyme assays. GDH activity was determined by the ammonia-dependent oxidation of either NADH or NADPH, whereas GOGAT was determined by the glutamine-dependent oxidation of either NADH or NADPH, which was followed spectrophotometrically at 340 nm in appropriate assay mixtures at room temperature by the procedure of Prusiner et al. (20, 26). GS was assayed by transferase activity as described by Shapiro and Stadtman (23). Transferase activity was determined by the amount of γ-glutamyl hydroxamate formed. *Rhizobium* strains contain two characteristic GSs, GSI and GSII. GSI and GSII activities were determined in crude extracts by using their different heat stabilities at 50 or 60°C, depending on the strain. GSII is heat labile, whereas GSI is heat stable. The procedure of heat treatment was carried out as described by Fuchs and Keister (13).

RESULTS

Influence of NaCl concentration on the content and composition of amino acid pools. The amino acid pool of *Rhizobium* sp. strain WR1001 grown at 0 mM NaCl was similar to those of other gram-negative bacteria (3, 4, 17, 26). Glutamate was the predominant amino acid, accounting for 41% of the total amino acid content (Table 1). Alanine and aspartate were the two other amino acids found to be abundant in the pool. Glutamine, valine, glycine, lysine, etc., were found in relatively small amounts. Furthermore, Table 1 shows that glutamate increased to extremely high levels with increasing NaCl added to the medium. Relative to controls grown in the absence of NaCl, the levels of glutamate in the intracellular pools for cells grown at 100, 200, 300, 400, and 500 mM increased 6.8-, 15.1-, 17.3-, 24.5-, and 34.4-fold, respectively. Glutamate accounted for 88% of the total amino acid pool when cells were grown at 500 mM NaCl. The amino acid analyzer did not separate glutamine, serine, and asparagine. The total value of these three amino acids increased about 17-fold for cells grown in 500 mM NaCl compared to 0 mM;

TABLE 1. Influence of medium NaCl concentrations on the intracellular free amino acid composition of *Rhizobium* sp. strain WR1001

Amino acid	Amino acid concn (μmol/g of cells [dry wt])[a] with NaCl concn of (mM):					
	0	100	200	300	400	500
Cys	1.08	1.14	1.57	1.53	1.65	1.61
Asp	2.86	5.49	6.80	7.16	10.72	11.64
Thr	0.38	0.46	0.45	0.64	0.39	0.38
Gln/Ser/Asn	0.71	1.25	3.55	4.41	6.12	12.04
Glu	8.39	56.81	126.96	145.16	205.60	288.49
Pro	ND[b]	0.99	0.83	ND	ND	ND
Gly	0.61	0.53	0.91	0.85	1.23	1.07
Ala	2.73	2.41	3.69	3.53	3.61	2.65
Val	0.56	0.71	0.68	0.56	0.56	0.54
Met	0.62	1.77	3.01	4.23	4.19	4.30
Leu	0.26	0.27	ND	ND	ND	ND
Tyr	ND	ND	0.22	0.25	0.28	ND
Phe	0.42	0.47	0.93	0.71	1.00	0.95
His	0.29	0.24	0.30	ND	0.24	ND
Lys	0.65	1.01	1.36	1.25	2.46	2.10
Arg	1.11	1.58	1.59	1.59	1.28	1.63
Total free amino acid	20.67	75.13	152.85	171.69	239.33	327.4
Glutamate portion of pool (%)	40.59	75.61	83.06	84.54	85.91	88.11

[a] Free amino acids were extracted by 5% TCA and determined by amino acid analyzer.
[b] ND, Not detectable (concentration less than 0.2 μmol/g of cells [dry weight]).

they represented about 3.7% of the total amino acid pool. As medium salt concentration was increased from 0 to 500 mM, aspartate, methionine, and lysine increased four-, seven-, and threefold, respectively. Other amino acids showed very little change. Figure 1 shows the effect of medium NaCl on the intracellular amino acid pool.

Proline was not detected in 5% TCA extract, either by the acid ninhydrin method before separation or by the individual amino acid analysis after separation by the column in an amino acid analyzer. Although it is one of the major intracellular amino acids in some microorganisms when grown in a high-salt medium (17, 18), proline does not appear to be an important amino acid with regard to response to NaCl in *Rhizobium* sp. strain WR1001.

GDH activity. Cell-free extracts of *Rhizobium* sp. strain WR1001 showed minute amounts of NADH-dependent GDH activity, but no NADPH-dependent GDH activity was observed. No significant change in activity occurred when glutamate was substituted for nitrate in the medium. The results are summarized in Table 2. In this study, we also included several other *Rhizobium* strains: two fast-growing strains, *R. meliloti* 2011 and *R. meliloti* 41; and two slow-growing, salt-sensitive strains, *R. japonicum* 110 and *Rhizobium* sp. strain CB756. There were great variations in GDH activities among these strains; *R. meliloti* 2011 had both NADH- and NADPH-dependent GDH activities when grown in either nitrate or glutamate medium. In *R. meliloti* 41, only NADH-dependent GDH activity was detectable. The two slow-growing *Rhizobium* strains, *R. japonicum* 110 and *Rhizobium* sp. strain CB756, did not grow in nitrate medium. When grown in glutamate, both NADH- and NADPH-dependent GDH activities were found in *Rhizobium* sp. strain CB756; only NADPH-dependent GDH activity was detectable in *R. japonicum* 110 (Table 2). We observed NADH oxidase activity in crude extracts in the absence of 2-ketoglutarate. When measuring GDH activity in the presence of 2-ketoglutarate, we subtracted the amount of NADH oxidized by NADH oxidase to make the determination.

Effect of in vitro KCl and NaCl concentrations on GDH activity. In several bacterial strains, Measures (17) found a 10-fold increase in NADPH-dependent GDH activity in vitro when stimulated by 500 mM KCl. The GDH activity varies greatly in *Rhizobia* spp., depending on the strain. Only *R. meliloti* 2011 was found to have a moderate amount of NADPH-dependent GDH activity. Therefore, we used a cell-free extract prepared from *R. meliloti* 2011 to test the effect of KCl on GDH activity (Table 3). There was a slight stimulation of enzyme activity at 100, 200, and 300 mM KCl. The enzyme activity was stable at 100 to 300 mM KCl and decreased by 20% at 300 to 500 mM KCl. In contrast, KCl at all concentrations from 100 to 500 mM appeared to inhibit the NADH-dependent GDH activity of *Rhizobium* sp. strain WR1001 when added to the

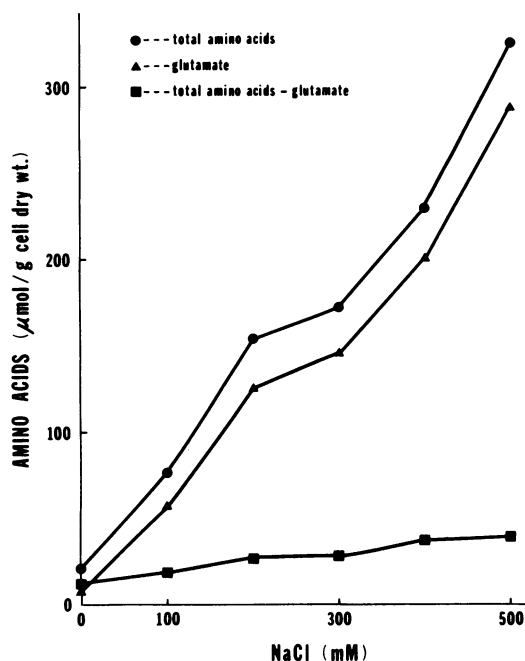

FIG. 1. Effect of medium NaCl concentrations on intracellular amino acid pools of *Rhizobium* sp. strain WR1001. Bacteria were grown in minimal nitrate-mannitol medium with the addition of 0 to 500 mM NaCl. Intracellular amino acids were extracted and analyzed by the method described.

enzyme assay mixture. NaCl had a similar effect on NADH-dependent GDH activity: it inhibited the enzyme activity at all concentrations tested (Table 3).

Effect of growth medium NaCl concentration on GDH activities. *Rhizobium* sp. strain WR1001 was capable of growth in medium containing 500 mM NaCl. We then examined whether NaCl in the growth medium would induce any GDH activity. Cell-free extracts were prepared from *Rhizobium* sp. strain WR1001 cultures grown in nitrate medium with salt concentrations ranging from 0 to 500 mM NaCl. There was no detectable NADPH-dependent GDH activity at any medium salt concentration. NADH-dependent GDH activity did not vary significantly with changes in medium salt concentration; it remained at the low level found in extracts from cultures grown on 0 mM NaCl.

GS and GOGAT activities. Since *Rhizobium* sp. strain WR1001 does not seem to have active enough GDH to fulfill a biosynthetic function of glutamate formation, we therefore examined the GS-GOGAT activities in *Rhizobium* sp. strain WR1001 and several other *Rhizobium* strains (Table 2). In all strains assayed, two GS activities were observed. The total GS activities of *Rhizobium* sp. strain WR1001 were very similar when either nitrate or glutamate was used as the nitrogen source. The nitrogen source appeared to affect the ratio of GSI to GSII. GSI activity was higher than that of GSII when grown in nitrate medium, but when glutamate was used as the nitrogen source, GSII activity was higher. The other *Rhizobium* strains used in this study have higher GSII than GSI activity in either nitrate or glutamate medium.

GOGAT activity varied greatly in *Rhizobium* strains (Table 2). *R. meliloti* 2011 had the highest NADPH-dependent GOGAT activity of any of the strains assayed when grown with nitrate-mannitol medium as the nitrogen source. The levels of both NADH- and NADPH-dependent GOGAT activities were much lower when grown in glutamate-mannitol medium. Small amounts of NADH- and NADPH-dependent GOGAT activities were found in *R. meliloti* 41, *R. japonicum* 110, and *Rhizobium* sp. strain

TABLE 2. GDH, GS, and GOGAT activities in *Rhizobium* strains

Strain	Nitrogen[a] source	GDH (nmol min^{-1} mg^{-1})		GS (nmol min^{-1} mg^{-1})[b]		GOGAT (nmol min^{-1} mg^{-1})	
		NADH	NADPH	GSI	GSII	NADH	NADPH
Rhizobium sp. strain WR1001	Nitrate	2.4	ND[c]	2,600	1,470	1.9	ND
	Glutamate	2.6	ND	2,000	2,260	2.9	ND
R. meliloti 2011	Nitrate	8.0	13.4	870	1,500	5.0	29.9
	Glutamate	17.1	29.0	870	1,530	2.7	6.3
R. meliloti 41	Nitrate	9.3	ND	1,200	2,040	5.4	7.5
	Glutamate	10.8	ND	810	1,150	3.9	4.8
R. japonicum 110	Glutamate	ND	1.2	1,910	2,080	10.0	2.8
Rhizobium sp. strain CB756	Glutamate	2.5	2.4	1,240	3,380	9.8	5.2

[a] Either glutamate or nitrate was added to the medium as the sole source of nitrogen. Glutamate was used at 0.09% (wt/vol) and adjusted to pH 7.0 with 1 N KOH.
[b] GS activities were assayed at pH 7.0.
[c] ND, Not detectable (enzyme activity less than 1 nmol min^{-1} mg^{-1}).

TABLE 3. Effect of in vitro salt concentrations on GDH activity

Salt concn (mM)	GDH (nmol min^{-1} mg^{-1})[a]		
	NADPH KCl (*R. meliloti* 2011)	NADH	
		KCl (*Rhizobium* sp. strain WR1001)	NaCl (*Rhizobium* sp. strain WR1001)
0	13.4	2.4	2.4
100	18.8	ND[b]	2.6
200	18.6	ND	1.5
300	16.6	ND	ND
400	12.7	ND	ND
500	10.3	ND	ND

[a] Enzyme extract prepared from cells grown in nitrate-mannitol medium.

[b] ND, Not detectable (enzyme activity less than 1 nmol min^{-1} mg^{-1}).

CB756. A very low level of NADH-dependent GOGAT activity was detected in *Rhizobium* sp. strain WR1001.

DISCUSSION

We followed the growth of *Rhizobium* sp. strain WR1001 grown in media containing different concentrations of NaCl. There was a slight stimulation of growth at 100 to 200 mM NaCl. The growth rate decreased as NaCl concentration increased from 300 to 500 mM. However, the bacterium could grow and survive in 500 mM NaCl. In contrast, most strains of *R. japonicum* were very sensitive to salt. Minimal medium containing 45 mM NaCl slows down the growth rate to a great extent in some variants of *R. japonicum* (27).

One of the goals of this study was to analyze the amino acid pool of *Rhizobium* sp. strain WR1001 grown in media containing different amounts of NaCl. Intracellular glutamate was found to increase rapidly in response to osmotic stress by NaCl. Glutamate has also been found to accumulate in several gram-negative bacteria by other investigators (17, 18, 26). Changing the osmolarity of the medium with NaCl did not increase intracellular proline content, although proline accumulation is important in many gram-positive bacteria, as well as some gram-negative bacteria. In general, bacterial species which accumulate proline are more salt tolerant than those which do not (17).

Measures (17) found a 10-fold increase of GDH activity in the direction of glutamate formation when 500 mM K^+ was added to the enzyme assay of cell-free extracts of *Escherichia coli, Klebsiella aerogenes, Bacillus subtilis,* and *Staphylococcus aureus*. Dendinger and Brenchley (9) demonstrated a twofold increase of NADPH-dependent GDH activity by 500 mM K^+ in *Salmonella typhimurium*, but the temperature-sensitive GDH from a mutant strain was inhibited severalfold by 500 mM KCl. We found only a slight stimulation of GDH activity at 100 to 300 mM KCl in cell-free extracts of *R. meliloti* 2011. The enzyme activity of NADPH-dependent GDH in *R. meliloti* 2011 was much lower than in the other bacteria.

Apparently, *Rhizobium* species have very diversified GDH activities. Our results of the several *Rhizobium* strains assayed confirmed the findings of other investigators. Osborn and Signer (19) found NADPH-dependent GDH activity in *R. meliloti* 2011. Ludwig (16) reported that *Rhizobium* sp. strain 32H1 did not have any detectable NADH- or NADPH-dependent GDH activity. Brown and Dilworth (5) observed both NADH- and NADPH-dependent GDH activities in various *Rhizobium* strains, and the activities varied considerably depending on the particular strain.

Darrow and Knotts (8) first demonstrated that *R. japonicum* 61A76 had two GS activities. Fuchs and Keister (13) have shown that bacterial strains in the *Rhizobiaceae* contain two characteristic GSs, GSI and GSII. GSI can undergo adenylation and is in other respects analogous to GS in *E. coli* and gram-negative bacteria. The new GS (GSII) was heat labile, and no evidence for adenylation was found. The two isoenzymes have been shown to be different in charge, sedimentation behavior, catalytic properties, and physiological regulation (7). *Rhizobium* sp. strain WR1001 showed two GS activities by heat inactivation. GSI is heat stable, and GSII is heat labile. Treatment of enzyme extract at 60°C for 15 min eliminates GSII activity. The heat stability of GSII resembles that of slow-growing *Rhizobium* spp.

Rhizobium sp. strain WR1001 can grow in minimal medium containing 3% NaCl. When it is stressed by high osmolarity in the growth medium, a pronounced elevation in intracellular concentration of L-glutamate was observed. Since the bacteria were grown in the absence of exogenously added amino acid, high concentrations of glutamate must be due to the net rate of synthesis. We examined the role of GDH in glutamate accumulation in *Rhizobium* sp. strain WR1001. Very minute amounts of NADH-dependent GDH activity were detected. The low specific activity of GDH suggests that this enzyme cannot fulfill the biosynthetic function to produce high concentrations of intracellular glutamate. Experimental data indicate that GS-GOGAT is the major pathway of ammonia assimilation in *Rhizobium* spp. (16, 19). It is likely that GS and GOGAT may be an important pathway for glutamate biosynthesis under salt stress.

Zablotowicz and Focht (31) observed that

plants inoculated with salt-tolerant *Rhizobium* sp. strain 176A28 recovered from drought stress to the same level of nitrogen fixation and nodulation as those that received adequate irrigation. However, plants inoculated with salt-sensitive *Rhizobium* strains failed to achieve the same level of recovery. The result implies that salt-tolerant *Rhizobium* spp. may be useful to maximize the nitrogen fixation potential in semiarid regions.

ACKNOWLEDGMENTS

We are grateful to G. Fuller, L. Sacks, and S. Schwimmer for critically reading the manuscript; R. Ludwig and M. Osburn for helpful discussion; and O. Chan for technical assistance.

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