MudPIT Profiling Reveals a Link between Anaerobic Metabolism and the Alkaline Adaptive Response of Listeria monocytogenes EGD-e

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Abstract

Listeria monocytogenes is a foodborne human pathogen capable of causing life-threatening disease in susceptible populations. Previous proteomic analysis we performed demonstrated that different strains of L. monocytogenes initiate a stringent response when subjected to alkaline growth conditions. Here, using multidimensional protein identification technology (MudPIT), we show that in L. monocytogenes EGD-e this response involves an energy shift to anaerobic pathways in response to the extracellular pH environment. Importantly we show that this supports a reduction in relative lag time following an abrupt transition to low oxygen tension culture conditions. This has important implications for the packaging of fresh and ready-to-eat foods under reduced oxygen conditions in environments where potential exists for alkaline adaptation.

Introduction

Listeria monocytogenes is a physiologically robust food-borne human pathogen. It is a facultative anaerobe, growing preferentially under microaerophilic conditions. During aerobic growth, energy generation in L. monocytogenes is achieved by both fermentation and aerobic respiration. Fermentation is homofermentative and is driven by substrate level phosphorylation (Embden-Meyerhof pathway). L. monocytogenes has a split citrate-cycle, it is capable of energy generation [1,2]. Aerobic respiration is characterised by the chemiosmotic movement of protons via ATP synthase as the final enzyme of an oxidative phosphorylation pathway [3,4]. The electron transport chain facilitating oxidative phosphorylation in L. monocytogenes is not fully defined, however a cytochrome has been characterised [3,6]. Under oxygen limited conditions, L. monocytogenes is able to generate energy by substrate-level phosphorylation alone (i.e. generation of ATP independent to electron acceptors or cellular respiration) and modulate its energy generation source (i.e. oxidative versus substrate level phosphorylation) in response to growth conditions has been described [5,6].

Results

Differentiation of Protein Expression

Differential protein expression in alkaline grown L. monocytogenes strain EGD-e was examined to uncover potential changes in protein expression in response to the extracellular pH environment. This involved an energy shift to anaerobic pathways and a shift in the source of energy generation following prolonged exposure to elevated concentrations of extracellular hydroxyl ions. This was tested by uncoupling oxidative phosphorylation using an ionophore. A working hypothesis was developed that alkaline grown L. monocytogenes strain EGD-e can modulate its source of energy generation for transition from aerobic to anaerobic growth and, consequently, would show decreased lag times if subsequently challenged by an abrupt shift to low oxygen tension. This could have important implications for the packaging of fresh and ready-to-eat foods under reduced oxygen conditions.

Materials and Methods

Bacterial Strain and Adaptation to Alkaline Culture Conditions

L. monocytogenes strain ATCC BAA-679 (EGD-e) was recovered from frozen (~−80°C) storage (Protect microbial preservation system; OXOID, Australia) and grown in 10 mL of Tris-buffered brain-heart infusion broth (CM225, ‘BHI’; OXOID, Australia), pH 7.5, incubated aerobically with shaking (30 rpm) at 37°C for 24 h.

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twenty hours. The strain was subcultured into fresh Tris-buffered BHI (pH 7.3), incubated as previously described, and the resulting starter culture used to inoculate subsequent cultures. Fresh 9.9 mL Tris-buffered BHI broths were prepared where the pH was adjusted to 7.3 or 9.0 (±0.2) through addition of 4 M NaOH (Sigma-Aldrich, Castle Hill, Australia). After autoclaving, the pH of both media (two×pH7.3, and two×pH9.0) was confirmed using an Orion 250A pH meter (Orion Research Inc, USA), and further adjusted using sterile NaOH or HCl if required. A 100 μL aliquot of the starter culture was transferred to the fresh broths and grown to exponential phase (OD600 = 0.4) aerobically with shaking at 37°C. 100 μL aliquots of these were transferred to fresh 9.9 mL BHI broths (with pH adjusted accordingly) and again incubated aerobically with shaking at 37°C. This was repeated three times to acclimatise the cultures to the growth conditions. The final pH for the pH 7.3 and 9.0 cultures was 7.1 and 8.9 respectively.

**MudPIT Analysis**

MudPIT was used to compare the protein expression profile of _L. monocytogenes_ strain EGD-e following adaptation to growth at pH9.0 (±0.2). Replicate 10 mL pH7.3 and 9.0 adapted cultures were prepared, incubated at 37°C, and harvested at late exponential phase (OD600 = 0.5–0.6; Figure 1) for proteomic analysis. The cultures were centrifuged at 10,000 ×_g_ for 10 min at 4°C and the supernatant was discarded. The pellets were resuspended in 500 μL of phosphate buffered saline (PBS; pH7.3 and pH9.0±0.2 respectively) and transferred into 1.5 mL Eppendorf Protein Lобind microcentrifuge tubes (Sigma-Aldrich, Castle Hill, NSW, Australia). The tubes were centrifuged at 14,000 ×_g_ for 5 min at 4°C and the PBS supernatant was discarded. The PBS wash was repeated twice. The cell pellets were frozen using liquid nitrogen then thawed on ice for ~15 min. Soluble proteins were extracted from the cell pellets using a Qproteome bacterial protein preparation kit (37900; Qiagen Pty. Ltd., Victoria, Australia) and approximate concentrations of the protein extracts was determined using a Pierce BCA Protein Assay kit (ThermoFisher Scientific, Victoria, Australia) according to manufacturer instructions. Volumes of protein extract containing ~50 μg of protein were transferred to clean Lobind microcentrifuge tubes, frozen with liquid nitrogen, and freeze-dried for ~8 h using a Dynavac mini ultra-cold vacuum freeze drier (Technolab, Kingston, Tasmania, Australia). The concentrated protein samples were digested with porcine trypsin (Sigma-Aldrich, Castle Hill, NSW, Australia) as described previously [13]. After digestion, the samples were centrifuged at 14000 ×_g_ for 5 min to remove insoluble material and 150 μL of the supernatants were transferred to high pressure liquid chromatography vials (Waters, USA).

MudPIT analysis was performed according to the method described by Delahunty and Yates [2005] using a ThermoFinnegian LTQ Orbitrap tandem mass spectrometer with a nano-electrospray ion source operated with a fragment-ion mass tolerance of 0.5 Dalton. Proteins in the sample were identified by matching the peptides predicted from the tandem mass spectra data against the complete _L. monocytogenes_ non-redundant database of the National Centre for Biotechnology Institute (NCBI) using the Computational Proteomics Analysis System (CPAS) Version 8.1 (www.labkey.org). Searches were semi-tryptic, with fixed modifications (cysteine carboxamidomethylation-57 Daltons) allowing no missed cleavages, and used the X!Tandem algorithm (www.thegpm.org/tandem/). Spectra counts within each sample were determined using TPP Xexpress Quantitation software (Version 2.1) in conjunction with X!Tandem. Functional assignment of protein identifications was predicted manually using The Institute for Genomic Research Comprehensive Microbial Resource (JCVI-CMR) (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?org = ntm01), GenoList _L. monocytogenes_ serovar 1/2a EGD-e database (Version 3) (http://genodbl.pasteur.fr/cgi-bin/WebObjects/GenoList.woa/wa/goToTaxRank?level = Listeria%20monocytogenes%20EGD-e), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/).

All searches were run through the Trans Proteomic Pipeline (TPP; Version 3.4) for statistical purposes. The TPP analysis utilised the “Peptide Prophet” and “Protein Prophet” algorithms as previously described [14] to enable the level of false positive peptide and protein identifications to be estimated and to generate a peptide and protein error rate. Identifications with an average peptide protein error rate (PER) of >0.3/1, and protein identifications assigned based on a single unique peptide, were not considered for further analysis. Relative protein abundances between growth conditions were determined using the spectra counting method [15]. Spectra counts were averaged between biological replicates and normalised to account for sampling depth [16]. Statistical significance of differences in spectra abundances for protein identifications between samples was assessed using a likelihood ratio test for independence (G-test) adjusted using the William’s correction (G_w) to reduce false positive rates [17,18]. Significance was assigned at p≤0.05 (G_w = 3.841). Only those protein identifications that met the filtering criteria (APPER and PER of >0.3/1) and that differed significantly from the control treatment are discussed.

**Uncoupling of Oxidative Phosphorylation**

Oxidative phosphorylation was uncoupled in alkaline adapted _L. monocytogenes_ EGD-e cells using the ionophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP; Sigma-Aldrich, Australia) [6,19]. Cultures were adapted to growth at pH7.3 and 9.0 as described previously. Replicate 10 mL cultures of each pH condition were prepared, incubated at 37°C, and CCCP was added to give a final concentration of 5 μM at mid-exponential growth phase (OD <0.5), measured automatically using a New Brunswick Scientific Low Oxygen Tension culture conditions. pH was adjusted to pH9.0 for each atmospheric condition) by injection through a polarographic DO probe (John Morris Scientific, Australia), was achieved by sparging N2 gas into the BHI media, and was maintained by a continual addition of N2 at a flow rate of 0.05 L min−1 prior to inoculation and during culture. pH was adjusted to 7.3 and 9.0 (±0.1) using sterile 5N NaOH and, if needed, 1N HCl. The cultures were grown at 37°C and were agitated at 50 revolutions per minute using an inbuilt motorised impeller. Replicate 250 mL Tris-buffered BHI cultures of _L. monocytogenes_ EGD-e were prepared, adapted to growth at pH7.3 and 9.0 as previously described and grown aerobically with shaking at 37°C. Following adaptation, an aliquot was collected at late exponential phase (OD =0.5–0.6) and was used to inoculate replicate microaerobic and aerobic BHI broths (two×pH7.3 and two×pH9.0 for each atmospheric condition) by injection through unidirectional rubber inoculation ports on the fermentors to give a starting OD of 0.3 (±0.05). Growth was monitored by viable

**Lag phase Determination Following an Abrupt Shift to Low Oxygen Tension**

Low oxygen tension culture conditions were prepared using 500 mL of BHI broth in a jacketed New Brunswick BioFlo/CelliGen 115 benchtop fermentor/bioreactor (John Morris Scientific, Australia). A dissolved oxygen (DO) concentration of ≈1% (≥0.5), measured automatically using a New Brunswick Scientific polarographic DO probe (John Morris Scientific, Australia), was achieved by sparging N2 gas into the BHI media, and was maintained by a continual addition of N2 at a flow rate of 0.05 L/min prior to inoculation and during culture. The cultures were grown at 37°C and were agitated at 50 revolutions per minute using an inbuilt motorised impeller. Replicate 250 mL Tris-buffered BHI cultures of _L. monocytogenes_ EGD-e were prepared, adapted to growth at pH7.3 and 9.0 as previously described and grown aerobically with shaking at 37°C. Following adaptation, an aliquot was collected at late exponential phase (OD =0.5–0.6) and was used to inoculate replicate microaerobic and aerobic BHI broths (two×pH7.3 and two×pH9.0 for each atmospheric condition) by injection through unidirectional rubber inoculation ports on the fermentors to give a starting OD of 0.3 (±0.05). Growth was monitored by viable
counting and the relative lag time of the cultures was determined as described by Mellefont et al. [20].

Results and Discussion

Proteomic Analysis of Alkaline Adapted L. monocytogenes EGD-e

L. monocytogenes EGD-e had a growth rate of 0.75 h⁻¹ when cultured aerobically at pH 9.0 compared to 0.84 h⁻¹ in the control system (pH7.3) and attained a mean maximum cell density of 9.6×10⁷ CFU mL⁻¹ and 6.7×10⁸ CFU mL⁻¹ at pH 9.0 and 7.3 respectively (Figure 1). A total of 1043 proteins were identified by MudPIT (approximately 37% of the L. monocytogenes EGD-e proteome). Application of the filtering criteria (average peptide prophet error rate (APPER) and protein error rate (PER) ≤ 0.3, unique peptides >1) reduced this to 534 high-confidence protein identifications with an average APPER of 0.096 and 0.085 and an average PER of 0.016 and 0.027 for the pH7.3 and 9.0 growth conditions respectively. These were assigned to functional categories based on the JCVI-CMR L. monocytogenes EGD-e functional ontology system (http://cmr.jcvi.org/cgi-bin/CMR/shared/RoleList.cgi) (Table S1). Statistical analysis showed that the abundance of 206 proteins differed significantly between treatments (G-test, p≤0.05; Table S1).

The protein profile of alkaline adapted L. monocytogenes EGD-e agreed with reported observations of pH homeostasis and an alkaline adapted cell physiology (Figure 2). Further, indirect evidence of a stringent-type response was observed and supported previous work investigating the L. monocytogenes proteome after adaptation to growth at alkaline pH (Figure 3; [11]). A part of this response involved a shift in energy metabolism from oxidative to substrate level phosphorylation (Figures 4 and 5).

Altering the flow of substrates through these pathways could serve a number of functions under alkaline conditions. The pentose phosphate shunt increases production of reducing equivalents (NADH) directed at fatty acid biosynthesis, the electron transport chain (ETC) and a wide range of other cellular processes. Increased proteins associated with fatty acid biosynthesis and degradation was observed in the present study; however, it was coupled to a decrease in other proteins also associated with biosynthesis of fatty acids (Table S1). While fatty acids are known to have a role in the pH tolerance response of L. monocytogenes, it is reported that the type, rather than number, of fatty acid is what imparts the protective effect [21]. On this basis the observed differences in protein abundances associated with fatty acid biosynthesis may reflect the type of fatty acids being produced and/or degraded, however this was not further investigated experimentally.

Importation of sugars via the phosphotransferase system (PTS) is shown to be important for buffering of the cell cytoplasm [22], while increasing substrates for glycolysis. Glycolysis, the pentose phosphate shunt and PTS system produce by-products that are associated with the electron transport chain. This multi-step energy generating system involves a number of protein components that transfer electrons from the initial NADH and succinate donors (generated by the pentose phosphate/glycolysis pathways, and the limited fatty acid degradation observed in the current study), culminating in energy production by an ATP synthase powered by a proton motive force [23]. However, in the present study, decreases in ubiquinone biosynthetic enzymes were
detected, along with decreased abundance of proteins associated with ATP-proton motive force (e.g. F0F1 ATPase subunits lmo0092, 0088, 2530, 2532 and 2528) and electron transport chain complexes one, two and five (e.g. NADH dehydrogenase lmo2638 and 2389, and fumarate reductase lmo0355; Figure 4 and 5). A decrease in proton motive force was supported by the increased expression of PTS system proteins (e.g. lmo0507, 1719, 2335, 1002 and 1003; Figure 4). Maintenance of intracellular pH in L. monocytogenes was shown by Shabala et al [22] to be coupled to two glucose transport systems: a low-affinity proton motive force-driven system and a high affinity PTS system. As such, should proton motive force be forcibly diminished it could be expected that proteins associated with the PTS-mediated glucose transport system would increase to compensate, as shown in Figure 4.

A diminished ATP-proton motive force would appear to oppose, to some extent, any cytoplasmic acidification process, as the proton pump (driven by the proton motive force) expels protons in the generation of energy (ATP) via an ATP synthase. Considering the decreased proton motive force and associated protein abundances, it is possible that under alkaline conditions the external pH is causing loss of protons, decreasing the flow back through the ATP synthase, and resulting in a net loss of protons from the cytoplasm (a reversal of the ATP synthase reaction). This would lead to a decrease in the proton motive force and could drive a decrease in ATP synthase in order to maximise proton retention within the cytoplasm. A decrease in ATP synthase activity in cells grown at alkaline pH has been demonstrated previously [24]. Consequently, we hypothesised that the mechanism driving the observed decrease in ATP synthase activity in alkaline adapted cells is a diminished proton motive force caused by the high cell surface pH (low proton concentration) resulting from the alkaline culture media. This concept is described in a microbial attachment model introduced by Hong and Brown [25]. In their model, they suggest that a charge-regulation effect may be induced by the environmental pH (attachment surface in their study) in proximity to the cell surface, resulting in a net

**Figure 2.** Protein groups identified in the current study previously associated with alkaline pH homeostasis [6,11,21,26,34,35,36,37,38]. Broad (A) and specific (B) functional grouping categories based on the JCVI-CMR L. monocytogenes EGD-e functional ontology system (http://cmr.jcvi.org/cgi-bin/CMR/shared/RoleList.cgi).

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migration of protons out of the cell. This migration of protons can shift the equilibrium of the ATP synthase phosphorylation reaction [24, 25, 26]. The associated deficit in energy (ATP) production may be offset by the observed increase in substrate level phosphorylation in the present study (Figure 5).

Figure 3. Evidence of stringent response (SR) induction in L. monocytogenes following alkaline adaptation. Characteristic expression of proteins previously associated with the bacterial SR was observed. A) Increased expression of the RelA synthase (a key enzyme involved in the bacterial SR), stress-related proteins (e.g. GroES, trigger factor and others), transcriptional repressor CodY, acetolactate synthase (AlsS), phosphocarrier protein (HPr), catabolite control protein A (CcpA) and decreased expression of elongation factors (EF’s) and the GTPase ObgE. Increased expression of the RelA synthase initiates production of the SR alarmone guanosine tetraphosphate (ppGpp) [39]. Chaperones stabilise proteins essential for survival during the SR and their expression can reportedly be induced by ObgE protein which is inversely correlated with ppGpp levels and cellular growth rate (as are the EF’s) [40, 41, 42, 43]. Increased expression of AlsS, associated with branched chain amino acid biosynthesis (BCAAS), reportedly has a role in both pH homeostasis and the bacterial SR representing a shunt of fermentation from end-product generation. Further, BCAAS is regulated by CcpA which, in turn, has been shown to complex with HPr to modulate many genes characteristic of the SR [44, 45]. Similarly, CodY has been shown to be induced as a part of the SR, is associated with regulation of >200 genes and transcriptional repression of CodY is increased in the presence of BCAA’s [42, 46, 47]. C) Aminoacyl-tRNA synthetase expression is decreased, correlating with the observed decrease in ribosomal proteins and growth rate at alkaline pH [40, 42].

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The combination of these mechanisms of acidification, including the charge regulation effect, could ultimately lead to a reducing environment within the cytoplasm, with a subsequent increase in reactive oxygen species through electron leakage [27]. This was suggested in the current study from the observation of significantly increased abundance of lmo1407 (pyruvate formate lyase; Table S1). This protein, generally associated with anaerobic metabolism, has been observed to increase under oxidative stress in the presence of increased reactive oxygen species [28]. Similarly, Listeria adhesion protein (lmo1634) was significantly increased at pH 9.0 and induction of this protein under anaerobic conditions has been described previously [29]. Given the evidence generated from the combined proteomics dataset we proposed that an energy generation shift towards fermentation was occurring during alkaline adaptation.

**Uncoupling of Oxidative Phosphorylation and Relative Lag Time after an Abrupt Shift to Low Oxygen Tension**

Proteomic analysis indicated that an energy shift induced in *L. monocytogenes* by prolonged exposure to alkaline culture conditions could support anaerobiosis and involved down-regulation of oxidative phosphorylation. To test whether oxidative phosphory-
Translation was reduced alkaline adapted and non-adapted cells were exposed to carbonyl \( m \)-chlorophenyl hydrazone (CCCP). CCCP is a chemical inhibitor of oxidative phosphorylation, achieved by uncoupling the proton gradient and consequently, interfering with ATP synthase’s ability to generate ATP [30]. Should alkaline adapted \( L. \) monocytogenes be more reliant on substrate-level rather than oxidative phosphorylation increased survival when exposed to CCCP would be expected.

Addition of CCCP inhibited growth at pH 7.3, while growth continued for the pH 9.0 grown cells (Figure 6A). This is consistent with a shift to predominantly substrate-level phosphorylation from oxidative phosphorylation and, when coupled with our proteomic findings, the transition to anaerobiosis. This conclusion was further supported by a significant decrease in expression of acetolactate decarboxylase (lmo1992; Figures 4 and 5), the final enzyme in the acetoin biosynthesis pathway and a metabolic indicator of anaerobic growth in \( L. \) monocytogenes [31]. Furthermore, acetoin was not detected in culture fluids of alkaline adapted \( L. \) monocytogenes EGD-e cells using a Voges-Proskauer method [32] but was at pH 7.3 (data not shown).

Importantly, relative lag time (RLT) following an abrupt shift to low oxygen tension (1% \( \sim 0.5 \)) supported a transition to anaerobiosis during alkaline adaptation, with reduced RLT for alkaline adapted \( L. \) monocytogenes EGD-e relative to non-adapted (pH 7.3) (Figure 6B). This is an important finding given that removal of air is a commonly applied food preservation hurdle, and indicates that alkaline adapted \( L. \) monocytogenes EGD-e may be capable of reaching dangerous numbers under such conditions faster than non-alkaline adapted cells.

Figure 5. Proteins associated with substrate level phosphorylation observed to be significantly increased (red font = increased, green font = decreased) following adaptation to growth at pH9.0. Sections of pathways where no proteins were identified are indicated with a double forward slash. KEGG enzyme classification numbers are shown. **Transport intermediate.
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Conclusions

MudPIT analysis indicated that alkaline pH homeostasis in \textit{L. monocytogenes} EGD-e results from multiple regulatory mechanisms driven by the necessity to increase cytoplasmic acidity, maintain cellular integrity, and minimize other physical effects imparted by the extracellular environment. A key component of this response involves direct cytoplasmic acidification through the production, retention and importation of polar or charged proteins, peptides and amino acids. Furthermore, systems are mobilized that facilitate stabilization of these and other regulatory proteins to prevent pH induced conformational changes that may lead to loss of function. This is coupled with an adaptive shift in energy metabolism that increases production of acidic by-products, ATP and reducing equivalents, to compensate for inhibition of other energy production pathways that are physically influenced by the extracellular pH environment. This includes restriction of the electron transport chain and inversion of the proton motive force to conserve protons that are being lost due to a charge-regulation effect, and to increase the pool of oxidized reducing equivalents.

Importantly, the modified physiology of alkaline adapted \textit{L. monocytogenes} matches that expected during anaerobiosis and allows for rapid growth (decreased lag time) following an abrupt shift to low oxygen tension. This is likely to be limited to very low oxygen tensions rather than strict anaerobiosis as increased recruitment of the aerobic Class Ia ribonucleotide reductase (RNR; system e.g. lmo2155) was evident in the alkaline adapted cells, possibly in an effort to ‘scavenge’ the limited oxygen available, while the abundance of anaerobic Class III RNR (e.g. lmo2079) while increased, did not differ significantly. Class III RNR is essential for growth of \textit{L. monocytogenes} under strict anaerobic conditions, and in \textit{L. monocytogenes} EGD-e, this protein is non-functional due to a deletion in a key catalytic site [33]. Results from this work suggest that \textit{L. monocytogenes} EGD-e is able to grow at very low oxygen tensions and may still be a suitable strain for experiments using such conditions.

This has important food safety implications, showing that alkaline adaptation in \textit{L. monocytogenes} is able to generate a phenotype capable of proliferating in very low oxygen tensions faster than non-adapted cells. This has relevance to food packaging procedures that employ the removal of oxygen as a growth limiting hurdle. Consequently, the combination of food packaged under low oxygen tension and the potential for \textit{L. monocytogenes} to become adapted to alkaline agents must be a consideration when assessing the risk of food contaminations by this organism. Further work is needed to investigate how this translates to the nutrient flux (including nutrient limitation) \textit{L. monocytogenes} likely encounters within food processing and production environments.

Supporting Information

Table S1 Proteins recovered from \textit{L. monocytogenes} EGD-e after adaptation to growth in BHI media with the pH adjusted to 7.3 and 9.0. PER = Protein Error Rate; APPER = Average Peptide Prophet Error Rate; UP = Unique Peptides. Functional assignment of protein identifications was predicted manually using The Institute for Genomic Research Comprehensive Microbial Resource (JCVI-CMR) (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?org=ntlm01). Significantly different protein abundances (G-test; p ≤0.05) are indicated with shading and their common names are shown.

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Author Contributions

Conceived and designed the experiments: RN JB TR. Performed the experiments: RN. Analyzed the data: RN MB. Contributed reagents/materials/analysis tools: JB TR MB. Wrote the paper: RN MB.
